Conference on Murine Leukemia

nci

MONOGRAPH 22

LIBRARY
AUG [illegible] 1974
NATIONAL INSTITUTES OF HEALTH

NATIONAL CANCER INSTITUTE MONOGRAPH 22

September 1966

Conference on Murine Leukemia

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL CANCER INSTITUTE, BETHESDA, MARYLAND

NATIONAL CANCER INSTITUTE MONOGRAPHS

KENNETH M. ENDICOTT, *Director, National Cancer Institute*

The proceedings of conferences and symposia dealing with cancer or closely related research fields and series of papers on specific subjects of importance to cancer research are presented in these monographs. Send an original, 1 carbon copy, and 2 sets of illustrations (1 mounted) to the Scientific Editor, National Cancer Institute, Bethesda, Maryland 20014. If there is a Conference Editor, manuscripts should be submitted to him.

Text: Double space all material. Block title and byline on right half of page. Official title or academic degree is optional. Align first word of text with first word of title. In typing, do *not* split a paragraph from one page to another—even if a large amount of blank space is left at the end of the last full paragraph on a page. Type tables on separate pages immediately after their description in text. Insert footnotes as separate paragraphs immediately after they are mentioned. Follow *Webster's International Dictionary* in matters of spelling, capitalization, punctuation, and hyphenation. Follow *Chemical Abstracts* for chemical terms.

References: Double space and number in order of citation in both text and reference page. Give author, title, journal, volume, first and last page numbers, and year. For book references, give chapter titles when referred to, and name and location of publisher.

Illustrations: Refer to graphs, charts, and line drawings as text-figures and number consecutively throughout text. Type legends in text immediately after paragraph in which text-figures are described. Make lettering of art uniform and large enough to be legible if reduced. Original drawings in black India ink or glossy prints are acceptable. Orient horizontally instead of vertically.

Photographs are printed at the end of each article. Refer to them as figures and number consecutively throughout text. Trim each photograph to show only important area. Maximum size of page for photographs is 5¼ × 6 inches. Mount and number one set on white cardboard, with as many figures to a page as possible. Reproduction is best when prints are from negatives of evenly illuminated subject in which details are sharp.

BOARD OF EDITORS

HOWARD B. ANDERVONT, *Scientific Editor*

JOHN C. BAILAR III, *Associate Editor*

GREGORY T. O'CONOR, *Associate Editor*

LAURENCE R. DRAPER, *Associate Editor*

SHERMAN M. WEISSMAN, *Associate Editor*

JACQUELINE J. K. WHANG, *Associate Editor*

EDITORIAL STAFF

DORIS M. CHANEY, *Managing Editor*

VIVIAN J. HESTON, *Assistant Managing Editor*

MARIE K. MURPHY, *Monograph Editor*

Assistant Editors: MERCEDES B. LA CHARITY, ALICE McA. LEE, and JOAN O'BRIEN

For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402. Price $6.25. Payment is required in advance, and check or money order should be made payable to the Superintendent of Documents.

CONFERENCE ON MURINE LEUKEMIA

Sponsored by
National Cancer Institute
National Institutes of Health
Bethesda, Maryland
and
Albert Einstein Medical Center
Philadelphia, Pennsylvania

Editors: Marvin A. Rich
and
John B. Moloney

CONFERENCE ON MURINE LEUKEMIA

Organizing Committee
MARVIN A. RICH, *Chairman*
JOHN B. MOLONEY
ROBERT E. STEVENSON

Acknowledgements

The Conference on Murine Leukemia was supported by the Virology Research Resources Branch, National Cancer Institute, National Institutes of Health. We are also indebted to the following organizations, whose support and cooperation have helped to make this Conference possible: Leukemia Society, Inc., New York, New York; Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania; American Cancer Society, Inc., New York; Damon Runyon Memorial Fund for Cancer Research, Inc., New York. Additional support was provided by: Bionetics Research Laboratories, Inc., Falls Church, Virginia; Carworth Farms, New City, New York; Hazleton Laboratories, Inc., Falls Church, Virginia, The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana; Research Laboratories, Merck Sharp & Dohme, West Point, Pennsylvania; Chas. Pfizer & Co., Inc., Maywood, New Jersey; Sandoz Pharmaceuticals, Hanover, New Jersey; Bio-Science Laboratories, Los Angeles, California; Ciba Pharmaceutical Company, Summit, New Jersey; Institute for Scientific Information, Philadelphia, Pennsylvania; Arthur D. Little, Inc., Cambridge, Massachusetts; Research Laboratories, Parke, Davis & Company, Ann Arbor, Michigan; Ivan Sorvall, Inc., Norwalk, Connecticut.

We acknowledge with sincere appreciation both the contributions and cooperation of all the participants. The Organizing Committee is indebted to Dr. Richard Siegler and Miss Phyllis Rosenblum for their excellent assistance in arranging the Conference. The editors are grateful to Miss Barbara Crowley for secretarial aid and to Miss Rosenblum for her invaluable assistance in organizing the manuscripts and discussion. The editors thank the editorial staff of the *Journal of the National Cancer Institute* for their assistance in preparing this Monograph.

PARTICIPANTS

Dr. Samuel J. Ajl, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*
Dr. T. Aoki, *Sloan-Kettering Institute for Cancer Research, New York, New York*
Dr. Arthur A. Axelrad, *Ontario Cancer Institute, Toronto, Canada*
Dr. Georges Barski, *Institut Gustave Roussy, Villejuif (Seine), France*
Dr. Robert Bases, *Albert Einstein College of Medicine, Bronx, New York*
Dr. Roy Bather, *University of Saskatchewan, Saskatoon, Canada*
Mrs. Dorothy Beard, *Duke Medical Center, Durham, North Carolina*
Dr. Joseph Beard, *Duke Medical Center, Durham, North Carolina*
Dr. Leonard Berwick, *University of Pennsylvania, Philadelphia, Pennsylvania*
Dr. L. Chieco-Bianchi, *Istituto di Anatomia ed Istologia Patologica, Università di Padova, Padova, Italy*
Dr. A. R. Bianco, *Universita di Napoli, Instituto di Patologia Speciale, Medica e Metrodologia Clinica, Naples, Italy*
Dr. Matthew H. Block, *University of Colorado Medical Center, Denver, Colorado*
Dr. George Blumenschein, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*
Dr. Charles Boone, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*
Dr. Daniel Boroff, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*
Dr. Robert G. Brackett, *Parke, Davis & Company, Detroit, Michigan*
Dr. Edward J. Breyere, *The American University, Washington, D.C.*
Dr. I. Brodsky, *Hahnemann Hospital, Philadelphia, Pennsylvania*
Dr. Eric Brown, *The Hektoen Institute for Medical Research, Cook County Hospital, Chicago, Illinois*
Dr. W. Ray Bryan, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*
Dr. Rita F. Buffett, *Roswell Park Memorial Institute, Buffalo, New York*
Dr. Michael A. Chirigos, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*
Dr. Natalie E. Cremer, *Department of Public Health, Berkeley, California*
Dr. Albert J. Dalton, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*
Dr. William Dameshek, *Pratt Clinic–New England Center Hospital, Boston, Massachusetts*
Mr. John Davenport, *Chas. Pfizer & Co., Inc., Maywood, New Jersey*
Dr. Vittorio Defendi, *The Wistar Institute, Philadelphia, Pennsylvania*
Dr. Etienne de Harven, *Sloan-Kettering Institute for Cancer Research, New York, New York*
Dr. Kenneth B. DeOme, *University of California, Berkeley, California*
Dr. Guy de-Thé, *Institut de Recherches sur le Cancer, 16 Bis, Avenue Vaillant-Couturier, Villejuif (Seine), France*
Dr. Leila Diamond, *The Wistar Institute, Philadelphia, Pennsylvania*
Dr. Leon Dmochowski, *M. D. Anderson Hospital, Houston, Texas*
Dr. Robert M. Dougherty, *State University of New York, Upstate Medical Center, Syracuse, New York*
Miss Yolande Dreyfuss, *Veterans Administration Hospital, Bronx, New York*
Dr. Virginia Dunkel, *Rutgers University, New Brunswick, New Jersey*

Dr. Thelma B. Dunn, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Maria Duran-Reynals, *Albert Einstein College of Medicine, Bronx, New York*

Dr. Ray Dutcher, *South Jersey Medical Research Foundation, Camden, New Jersey*

Dr. June East, *Imperial Cancer Research Fund, London, England*

Dr. Dorothy G. Feldman, *Veterans Administration Hospital, Bronx, New York*

Dr. Mary A. Fink, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Luciano Fiore-Donati, *Istituto di Anatomia ed Istologia Patologica, Università di Padova, Padova, Italy*

Dr. Marion Fogel, *Weizmann Institute of Science, Rehovoth, Israel*

Dr. Leonard Freed, *City Hospital, St. Petersburg, Florida*

Dr. Herman Friedman, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Charlotte Friend, *Sloan-Kettering Institute for Cancer Research, New York, New York*

Dr. Abraham Frumin, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Isamu Fukuma, *Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania*

Dr. Jacob Furth, *College of Physicians and Surgeons of Columbia University, New York, New York*

Dr. Paul Gerber, *Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland*

Dr. John P. Glynn, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Abraham Goldin, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Herman Goldner, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Arnold Graffi, *German Academy of Sciences, Berlin, Germany*

Dr. Ludwik Gross, *Veterans Administration Hospital, Bronx, New York*

Dr. M. Gruenstein, *Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania*

Dr. Françoise Haguenau, *College of France, Paris, France*

Dr. Jennifer Harvey, *M. D. Anderson Hospital, Houston, Texas*

Mr. Martin I. Hassner, *Leukemia Society, Inc., New York, New York*

Dr. Ursula Heine, *Duke Medical Center, Durham, North Carolina*

Dr. Maurice R. Hilleman, *Merck Institute for Therapeutic Research, West Point, Pennsylvania*

Dr. Robert Holdenried, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Noriaki Ida, *Toyo Kogyo Hospital, Hiroshima, Japan*

Dr. Harry L. Ioachim, *College of Physicians and Surgeons of Columbia University, New York, New York*

Dr. Laird Jackson, *Jefferson Medical College, Philadelphia, Pennsylvania*

Dr. Erling Jensen, *Chas. Pfizer & Co., Inc., Maywood, New Jersey*

Dr. Albert Kaplan, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Martin M. Kaplan, *World Health Organization, Geneva, Switzerland*

Mr. S. Kessler, *Carcinogenesis Abstracts, Philadelphia, Pennsylvania*

Dr. Werner Kirsten, *University of Chicago, Chicago, Illinois*

Dr. George Klein, *Karolinska Institute, Stockholm, Sweden*

Dr. Hilary Koprowski, *The Wistar Institute, Philadelphia, Pennsylvania*

Dr. Edward Klavitz, *Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania*

Dr. John Landon, *Bionetics Research Laboratories, Inc., Falls Church, Virginia*

Dr. Edward Larkin, *South Jersey Medical Research Foundation, Camden, New Jersey*

Dr. Raymond Latarjet, *Pasteur Institut, Paris, France*

Dr. Lloyd W. Law, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. J. P. Levy, *University of Paris, Institute of Research of the Faculty of Medicine, Paris, France*

Dr. Miriam Lieberman, *Stanford University School of Medicine, Palo Alto, California*

Dr. Frank Lilly, *Albert Einstein College of Medicine, New York, New York*

Dr. Fu Hai Lin, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Samuel Luborsky, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Lauri Luoto, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Robert A. Manaker, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Richard March, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Robert R. Marshak, *University of Pennsylvania, Philadelphia, Pennsylvania*

Dr. Edmund B. Masurovsky, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Sami A. Mayyasi, *Chas. Pfizer & Co., Inc., Maywood, New Jersey*

Dr. Donald Metcalf, *Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Victoria, Australia*

Dr. Edwin A. Mirand, *Roswell Park Memorial Institute, Buffalo, New York*

Dr. John B. Moloney, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. William C. Moloney, *Boston City Hospital, Boston, Massachusetts*

Dr. Peter T. Mora, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. William H. Murphy, *The University of Michigan Medical School, Ann Arbor, Michigan*

Dr. Timothy E. O'Connor, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Stanley R. Opler, *Lenox Hill Hospital, New York, New York*

Dr. Stephen Oroszlan, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Frank Padgett, *Indiana University Medical Center, Indianapolis, Indiana*

Dr. Arthur Pallotta, *Bionetics Research Laboratories, Inc., Falls Church, Virginia*

Dr. John C. Parker, *Microbiological Associates, Inc., Bethesda, Maryland*

Dr. Kalman Perk, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Roman Pienta, *Rutgers University, New Brunswick, New Jersey*

Dr. Donald Pinkel, *St. Jude Children's Research Hospital, Memphis, Tennessee*

Dr. Richard Pledger, *Hazleton Laboratories, Inc., Falls Church, Virginia*

Dr. Giancarlo Rabotti, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Henry Rappaport, *University of Chicago, Chicago, Illinois*

Dr. Frank J. Rauscher, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Louis Recher, *M.D. Anderson Hospital, Houston, Texas*

Dr. Arnold E. Reif, *Boston City Hospital, Boston, Massachusetts*

Dr. James Reyniers, *Germfree Life Research Center, Tampa, Florida*

Dr. Rodolfo Ribacchi, *Division of Cancer Research, University of Perugia, Perugia, Italy*

Dr. Marvin A. Rich, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Giovanni B. Rossi, *Sloan-Kettering Institute for Cancer Research, New York, New York*

Dr. Wallace P. Rowe, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Leo Sachs, *Weizmann Institute of Science, Rehovoth, Israel*

Dr. Guy Sainte-Marie, *The University of Western Ontario, Cancer Research Laboratory, London, Canada*

Dr. M. H. Salaman, *The London Hospital Medical College, University of London, London, England*

Dr. Frances T. Sanel, *College of Physicians and Surgeons, Francis Delafield Hospital, New York, New York*

Dr. Leonard D. Saslaw, *Microbiological Associates, Inc., Bethesda, Maryland*

Dr. Steven O. Schwartz, *The Hektoen Institute for Medical Research, Cook County Hospital, Chicago, Illinois*

Dr. Florence Seibert, *Mound Park Hospital, St. Petersburg, Florida*

Dr. David A. Shachat, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. Michael B. Shimkin, *Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania*

Dr. Louis Sibal, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. B. V. Siegel, *University of Oregon Medical School, Portland, Oregon*

Dr. Richard Siegler, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

Dr. Joseph Sinkovics, *M.D. Anderson Hospital, Houston, Texas*

Dr. John Smith, *St. Jude Children's Research Hospital, Memphis, Tennessee*

Dr. Philip G. Stansly, *Detroit Institute of Cancer Research, Detroit, Michigan*

Dr. Robert E. Stevenson, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Dr. John A. Sykes, *M.D. Anderson Hospital, Houston, Texas*

Dr. Albert Tannenbaum, *Michael Reese Hospital and Medical Center, Chicago, Illinois*

Dr. Judith R. Tennant, *Sloan-Kettering Institute for Cancer Research, New York, New York*

Dr. Viliam Thurzo, *Cancer Research Institute, Bratislava, Czechoslovakia*

Dr. R. C. Ting, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Mr. Irving Toplin, *Chas. Pfizer & Co., Inc., Maywood, New Jersey*

Dr. Bela Toth, *The Chicago Medical School, Chicago, Illinois*

Dr. John J. Trentin, *Baylor Medical School, Houston, Texas*

Dr. R. L. Tyndall, *Oak Ridge National Laboratory, Oak Ridge, Tennessee*

Dr. Arthur C. Upton, *Oak Ridge National Laboratory, Oak Ridge, Tennessee*

Dr. Lee Vernon, *Hazleton Laboratories, Inc., Falls Church, Virginia*

Dr. H. Wanebo, *Sloan-Kettering Institute for Cancer Research, New York, New York*

Dr. Louis R. Wasserman, *Mt. Sinai Hospital, New York, New York*

Dr. George H. Werner, *Rhone-Poulenc, Vitry sur Seine, France*

Dr. E. Frederick Wheelock, *Western Reserve University, School of Medicine, Cleveland, Ohio*

Dr. Roger Wilsnack, *Baltimore Biological Laboratories, Baltimore, Maryland*

Dr. Isidore Wodinsky, *Arthur D. Little, Inc., Cambridge, Massachusetts*

Dr. Bruce Wright, *Chas. Pfizer & Co., Inc., Maywood, New Jersey*

Dr. Nobuto Yamamoto, *Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania*

Dr. Tokichi Yumoto, *M.D. Anderson Hospital, Houston, Texas*

Dr. Kiyozo Yoshida, *St. Jude Children's Research Hospital, Memphis, Tennessee*

Dr. Robert Ziegel, *National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Miss Phyllis Rosenblum, Conference Secretary, *Albert Einstein Medical Center, Philadelphia, Pennsylvania*

TABLE OF CONTENTS

General Session

Chairman: Robert E. Stevenson

Page

The Mechanism of Leukemogenesis by the Rauscher Leukemia Virus
Dov H. Pluznik, Leo Sachs, and Peretz Resnitzky ---------------- 3

Tissue Culture and Serologic Studies of Mouse Leukemia Viruses
Wallace P. Rowe, Janet W. Hartley, and Worth I. Capps---------- 15

Experiments on the Hematologic Diversification of Viral Mouse Leukemias
A. Graffi, F. Fey, and T. Schramm------------------------------ 21

Descriptive Summary on Breast Tumors and Leukemia Induced in Female Wistar Rats Receiving Gastric Instillations of 3-Methylcholanthrene
M. B. Shimkin, M. Acuff, D. Thatcher, and M. Gruenstein--------- 33

Some Experimental Evidence for the Influence of Genetic Factors on Viral Leukemogenesis
Judith R. Tennant and George D. Snell------------------------- 61

Inhibition of Virus-Induced Leukemia in Mice by a Nontumor Virus
E. Frederick Wheelock-- 73

Morphology and Development of Murine Leukemia Viruses

Chairman: Leon Dmochowski

Origin of the Viremia in Murine Leukemia
Etienne de Harven and Charlotte Friend------------------------ 79

Morphology and Development of Some Murine Leukemia Viruses
Tokichi Yumoto, Louis Recher, John A. Sykes, and Leon Dmochowski- 107

A Virus-Induced Rhabdomyosarcoma of Mice
John B. Moloney (with the assistance of E. G. Jenkins and H. Sims)--- 139

An Electron Microscopic Study of a Virus-Induced Murine Sarcoma (Moloney)
Albert J. Dalton-- 143

Association of Enzymes: Adenosinetriphosphatase and Alkaline Phosphatase With the Virions of Murine Leukemias
Guy de-Thé-- 169

Isolation of the Nucleic Acid of Rauscher Murine Leukemia Virus
Peter T. Mora, Vivian W. McFarland, and Samuel W. Luborsky---- 191

Murine Leukemia Viruses: Rupture With Ether and Detergents to Subviral Constituents
Timothy E. O'Connor, Frank J. Rauscher, Guy de-Thé, Mary A. Fink, and Paul Gerber-- 205

Page

Studies on Leukemia in Mice Associated With a Transplantable Methylcholanthrene-Induced Hepatoma
L. Dmochowski, T. Rogers, A. G. Liebelt, R. A. Liebelt, and J. A. Sykes 223

Observations on the Morphology of a Murine Leukemia Virus (Rauscher) Propagated in Tissue Culture
Robert F. Zeigel, Richard L. Tyndall, Timothy E. O'Connor, Ernestine Teeter, and Barbara V. Allen 227

Viral, Chemical, and Radiation-Induced Murine Leukemia

Chairman: Joseph W. Beard

Transmission Studies of a Leukemogenic Virus, MLV, in Mice
L. W. Law 267

Several Aspects of Vertical Transmission of Moloney Virus
Noriaki Ida, Arimitsu Fukuhara, and Yasuhiro Ohba 287

Studies With Malignant Lymphomas: Possible Interaction Problems Between Chemical and Viral-Inducing Agents
Bela Toth and Philippe Shubik 313

Observations on Viral, Chemical, and Radiation-Induced Myeloid and Lymphoid Leukemias in RF Mice
A. C. Upton, V. K. Jenkins, H. E. Walburg, Jr., R. L. Tyndall, J. W. Conklin, and N. Wald 329

Occurrence of Low Leukemogenic but Immunizing Mouse Leukemia Virus in Tissue Culture
Joseph G. Sinkovics, Barbara A. Bertin, and Clifton D. Howe 349

Infective and Noninfective Viral Murine Leukemias
W. H. Kirsten, L. A. Mayer, and C. W. Welander 369

Antigenic Study on the Friend, Moloney, and Rauscher Viruses With the Electron Microscope Agglutination Test
Sami A. Mayyasi, George Schidlovsky, Laurette M. Bulfone, and Nan L. Clifford 379

Enhancing Effect of Chemical Carcinogens on Experimental Viral Infection: Its Significance and Probable Mechanism
Maria L. Duran-Reynals 389

Induction of a Leukemogenic Agent by a Chemical Carcinogen in Inbred Mice
L. A. Zilber and Z. A. Postnikova 397

Murine Leukemia Viruses—Their Differentiation and Classification

Chairman: John B. Moloney

Are the Common Forms of Spontaneous and Induced Leukemia and Lymphomas in Mice Caused by a Single Virus?
Ludwik Gross 407

Page

Murine Leukemia and Its Viruses
MARVIN A. RICH .... 425 ✓

A Comparison by Several Techniques of the Antibody Prepared in Various Species Against the Rauscher Murine Leukemia Virus
MARY ALEXANDER FINK, CAROLYN A. COWLES, MICHAEL A. CHIRIGOS, and J. MESSORE .... 439 ✓

Serological Analysis of Leukemia Antigens of the Mouse
TADAO AOKI, LLOYD J. OLD, and EDWARD A. BOYSE 449

Further Studies on the Biological Relationship of Murine Leukemia Viruses and on Kidney Lesions of Mice With Leukemia Induced by These Viruses
LOUIS RECHER, TOSHIO TANAKA, JOHN A. SYKES, TOKICHI YUMOTO, GABRIEL SEMAN, LANDRUM YOUNG, and LEON DMOCHOWSKI 459

Antigenic Behavior of Moloney Virus-Induced Lymphomas in Mice
GEORGE KLEIN and EVA KLEIN 481

Erythropoietic Response of Animals Infected With Various Strains of Friend Virus
EDWIN A. MIRAND 483

Erythrocytic Maturation *In Vitro* of Murine (Friend) Virus-Induced Leukemic Cells
C. FRIEND, M. C. PATULEIA, and E. DE HARVEN 505

The Thymus in Leukemogenesis

Chairman: JACOB FURTH

Pathogenesis of Murine Leukemia
RICHARD SIEGLER and MARVIN A. RICH 525

Lymphoid Tumor Induction by Mouse Thymocytes Infected *In Vitro* With Radiation Leukemia Virus
MIRIAM LIEBERMAN and HENRY S. KAPLAN 549

Prelymphomatous or Preleukemic State in Mice: Relation to Human Preleukemia
MATTHEW BLOCK 559

Analysis of the Role of the Thymus in Leukemia Development With the Use of Thymectomy and Thymus Grafts
D. METCALF, M. WIADROWSKI, and R. BRADLEY 571

Studies on Thymus-Dependent Mechanisms of Mouse Leukemogenesis by Graffi Virus
LUCIANO FIORE-DONATI, LUIGI CHIECO-BIANCHI, GIUSEPPE TRIDENTE, and NATALE PENNELLI 587

The Thymus Autoimmunity and Malignancy in New Zealand Black Mice
JUNE EAST and MARIA A. B. DE SOUSA 605

General Session

Chairman: Frank J. Rauscher

Page

Genetic Control of Susceptibility to Friend Leukemia Virus in Mice: Studies With the Spleen Focus Assay Method
A. Axelrad 619

The Histocompatibility-2 Locus and Susceptibility to Tumor Induction
Frank Lilly 631

Response of Rauscher Virus to Drug and Immune Serum Therapy *In Vivo*
Michael A. Chirigos, Estelle D. Luber, Srikrishna Vadlamudi, and Mary A. Fink 643

Protective Effect of Specific Immunization in Rauscher Leukemia
G. Barski and J. K. Youn 659 ✓

Studies of Moloney and Rauscher Leukemia Viruses in Cell Cultures by Immunofluorescence
Donald Pinkel, Kiyozo Yoshida, and Kirby Smith 671

Attenuation of the Rauscher Murine Leukemia Virus Through Serial Passages in Tissue Culture
Bruce S. Wright and Jennie C. Lasfargues 685

Leukemia Virus Release in Chemically or Physically Induced Lymphomas in BALB/c Mice
Rodolfo Ribacchi and Gaetano Giraldo 701

GENERAL SESSION

Chairman: Robert E. Stevenson

The Mechanism of Leukemogenesis by the Rauscher Leukemia Virus[1, 2]

Dov H. Pluznik, Leo Sachs, *and* Peretz Resnitzky,[3, 4] *Section of Genetics, Weizmann Institute of Science, and Laboratory of Blood Morphology and Cytology, Kaplan Hospital, Rehovoth, Israel*

TWO questions relating to the mechanism of viral leukemogenesis are: 1) Can cloned lines of virus induce more than one type of leukemia, and 2) are stem cells the target cells for the virus, or are they cells that have already undergone some degree of cell differentiation?

The present experiments were undertaken to answer these 2 questions. The virus used was the Rauscher leukemia virus (RLV), which has been found to induce a rapid erythroid leukemia and to cause the development of lymphoid leukemia in mice surviving the erythrocytopoiesis (*1–3*). The experiments to be presented are thus concerned with: 1) the types of leukemia induced by 14 cloned lines of RLV prepared by 3 successive single colony isolations from virus-induced cell colonies in the spleen (*4*), and 2) the use of hypoxia as an erythropoietic stimulant and of polycythemia as a suppressor of erythropoiesis (*5*) to determine the nature of the target cells for the erythroid leukemia induced by RLV.

MATERIALS AND METHODS

Virus stocks and titration.—The stocks of RLV were obtained from spleen extracts from leukemic mice, passages 262 and 856, kindly supplied by Dr. F. J. Rauscher, National Cancer Institute, Bethesda, Maryland, and from plasma from leukemic SWR mice after 62 to 109 animal passages in our laboratory. The virus was titered by the spleen colony assay

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by Public Health Service research grant CA-05266 from the National Cancer Institute.

[3] Kaplan Hospital.

[4] We are indebted to Mr. Mordechai, Ben Ezra, and Mr. Aryeh Goldenberg for skillful technical assistance.

after intravenous inoculation into adult mice, as described previously (*4*). The virus titers are given in colony-forming units (CFU), and colonies were counted at 7 days after virus inoculation. The same method of titration has also been used for the Friend virus (*6*).

Isolation of virus from single colonies.—Virus stocks were diluted in a phosphate-buffered saline (PBS) so as to give about 5 colonies per spleen. Eight days after virus inoculation, the spleens were removed and placed in petri dishes, containing PBS, on a bacterial colony counter lit from all sides. The colonies in the spleen could be seen even without fixation under proper illumination.

Single colonies were isolated from spleens with 5 to 8 colonies, and only well-separated colonies located on the edge of the spleen were used. Each isolated colony was passed with 0.5 ml PBS in a syringe through a #27 needle, and the cell suspension was sonically vibrated at 9000 cycles per second for 3 minutes in a Raytheon 200 ×, 10 kc magnetostrictive oscillator. The supernatant obtained after centrifugation at 650 × g for 15 minutes was used as the virus from a single colony. On inoculation, such a virus suspension from a single colony gave about 5 to 10 CFU. Pieces from the edge of the spleen with no visible colonies were isolated and treated in the same way as the isolated colonies. No colonies were obtained after inoculation of extracts from these control areas.

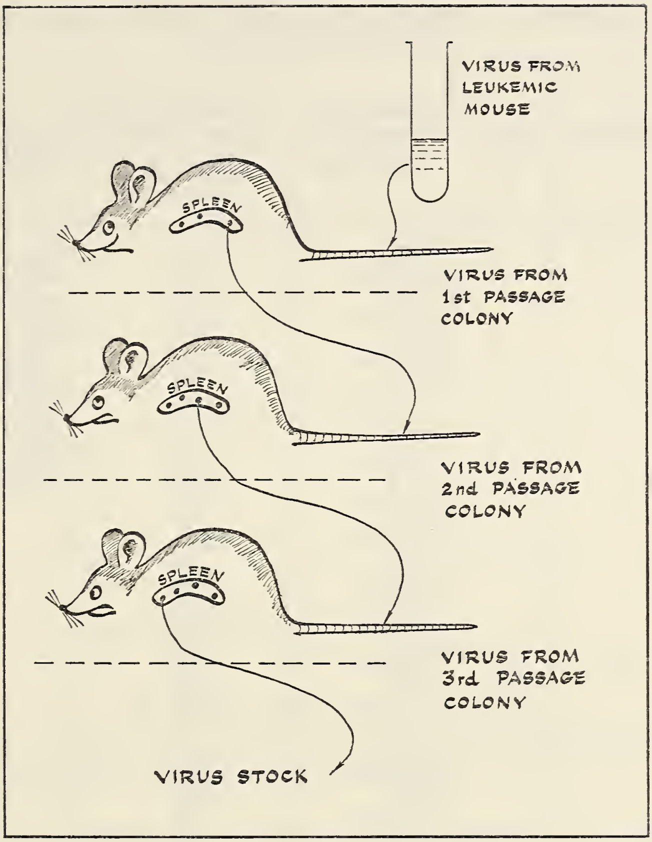
Production of hypoxia and polycythemia.—Hypoxia was produced by dilution of air with nitrogen, volume per volume, to give 10.5% ± 0.5% oxygen. The oxygen concentration was checked daily with a Beckman oxygen analyzer (model D2). For the experiments with hypoxia, the mice were injected with virus immediately after removal from 10.5% oxygen to the normal room atmosphere.

Polycythemia was produced by an extended period of exposure to hypoxia (*7*). For the experiments with polycythemia, the mice were kept in hypoxia for 15 days, and then in the normal room atmosphere for another 5 days, before they were injected with virus. Polycythemia in these mice was checked by measuring the hematocrit, and animals with hematocrits of less than 65% were excluded. The experiments with hypoxia and polycythemia were all carried out with 5- to 8-week-old SWR mice, and in all experiments the mice were kept in the normal room atmosphere after virus injection.

RESULTS

Types of Leukemia Induced by Cloned Lines of Virus

Fourteen lines of virus, derived from 3 virus stocks, were used to determine the types of leukemia induced by cloned lines of RLV. The virus of each line was subjected to 3 successive single colony isolations, as shown in text-figure 1. Virus stocks were made from the third passage


VIRUS FROM LEUKEMIC MOUSE
SPLEEN
VIRUS FROM 1st PASSAGE COLONY
SPLEEN
VIRUS FROM 2nd PASSAGE COLONY
SPLEEN
VIRUS FROM 3rd PASSAGE COLONY
VIRUS STOCK

TEXT-FIGURE 1.—Procedure used for the production of cloned lines of RLV by 3 successive single colony isolations.

colonies by 2 successive intravenous inoculations and the collection of plasma. Eleven lines were cloned in SWR mice and 3 in BALB/c. All 14 lines were tested for the types of leukemia in SWR and BALB/c male and female mice by intravenous inoculation of 1 to 10 CFU per mouse.

Of a total of 528 inoculated mice, 460 died with splenomegaly less than 3 months after virus inoculation. The 68 mice surviving more than 3 months were diagnosed by cell smears made from peripheral blood, spleen, liver, lymph nodes, bone marrow, and thymus. The cell smears were made up to 5 months after virus inoculation. Lymphatic leukemias were not found during this observation period. There were mice surviving more than 3 months with all the cloned lines of RLV, and all these mice had enlarged spleens and developed both erythroid and myeloid leukemia (table 1).[5] The detailed diagnosis of these leukemias will be published

---

[5] Since this paper was presented, the induction of myeloid leukemia by RLV has also been reported by Boiron *et al.* J Nat Cancer Inst 35 : 865–884, 1965.

TABLE 1.—Induction of erythroid and myeloid leukemia by 14 cloned lines of Rauscher leukemia virus

Origin of lines*	Line No.	Mouse strain tested for types of leukemia†	Number of mice surviving more than 3 months / Number of mice inoculated	Types of leukemia in mice surviving more than 3 months‡
Spleen extract, passage 262, obtained from Dr. F. J. Rauscher	1	SWR	3/24	Each mouse surviving more than 3 months developed both erythroid and myeloid leukemia
		BALB/c	4/14	
	2	SWR	0/24	
		BALB/c	5/14	
	3	SWR	8/24	
		BALB/c	4/24	
	4	SWR	1/24	
		BALB/c	7/24	
Plasma from SWR mice after 62 passages in our laboratory	5	SWR	1/24	
		BALB/c	2/14	
	6	SWR	0/24	
		BALB/c	2/14	
	7	SWR	2/24	
		BALB/c	2/14	
	8	SWR	3/24	
		BALB/c	5/14	
	9	SWR	0/24	
		BALB/c	3/14	
	10	SWR	0/24	
		BALB/c	4/14	
	11	SWR	0/24	
		BALB/c	3/14	
Spleen extract, passage 856, obtained from Dr. F. J. Rauscher	12	SWR	0/15	
		BALB/c	1/15	
	13	SWR	3/15	
		BALB/c	2/15	
	14	SWR	0/15	
		BALB/c	3/15	
		Total No. of mice	68/528	

*Lines 1 to 11 cloned in SWR mice, and lines 12 to 14 cloned in BALB/c mice.

†Four- to 6-week-old mice were inoculated intravenously with 1 to 10 CFU per mouse.

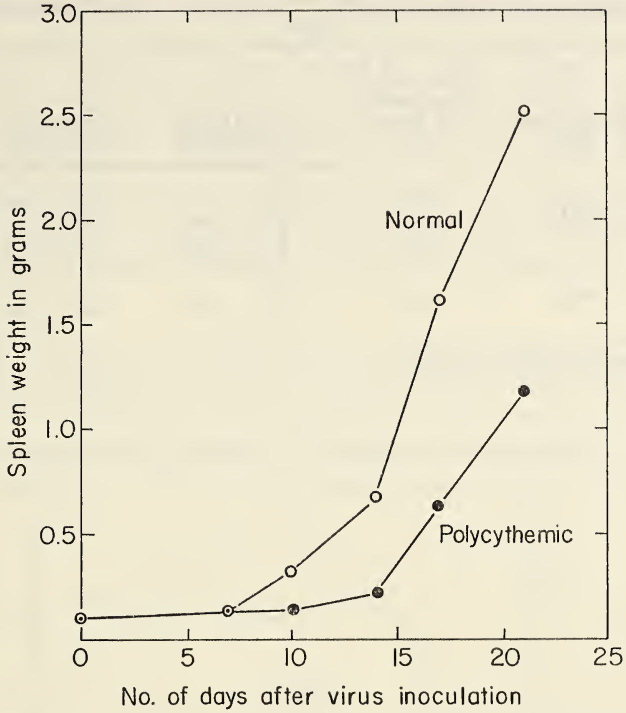
‡All mice surviving less than 3 months died with splenomegaly. Leukemias in mice surviving more than 3 months were diagnosed by cell smears made from peripheral blood, spleen, liver, lymph nodes, thymus, and bone marrow. Smears were made at 3 to 5 months after virus inoculation. At this time all mice had enlarged spleens.

elsewhere. These 2 types of leukemia were not observed in control mice of the same age.

Nature of the Erythroid Target Cells

Effect of polycythemia

When erythropoiesis was suppressed by polycythemia, inoculation of RVL resulted in a reduction in the number of colonies in the spleen



Text-figure 2.—Spleen weight at different times after virus inoculation in normal and polycythemic mice. Average results from 5 mice.

(table 2). At 7 days after virus inoculation there was an average of 3 colonies in the polycythemic animals compared to 15 colonies in the controls, and at 10 and 14 days the colonies were confluent in the controls but not in the polycythemic mice. At 17 and 21 days after virus inoculation the colonies in both groups were confluent. The hematocrit of the polycythemic mice was over 60% up to 10 days after virus inoculation, and then decreased to about 50% between 14 to 17 days. A comparison of spleen weights (text-fig. 2) also showed an inhibition of increase in spleen weight in the polycythemic mice up to about 14 days.

Table 2.—Number of colonies per spleen in normal and polycythemic SWR mice*

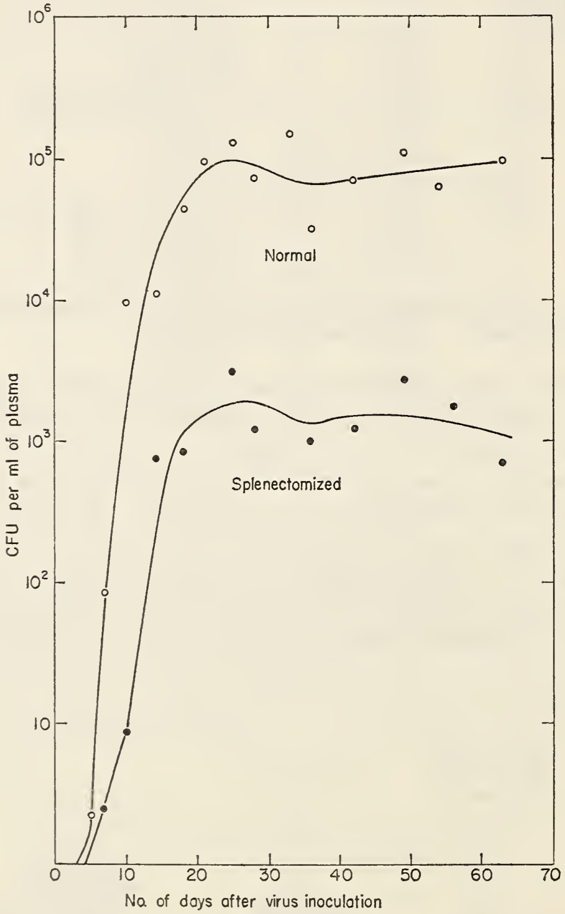
Number of days after virus inoculation	Polycythemic mice	Normal mice
7	3	15
10	1	Confluent†
14	5	Confluent
17	Confluent	Confluent
21	Confluent	Confluent

*Average results from 5 spleens.
†Confluent = >60 colonies per spleen.

TABLE 3.—Virus titer in plasma of normal and polycythemic SWR mice*

Experiment No.	Number of days after virus inoculation	Polycythemic mice CFU/ml	Normal mice CFU/ml
381	7	5	1.1×10^3
	10	10	2.5×10^4
	14	5.6×10^3	9.2×10^4
	17	1.7×10^4	1.8×10^5
405	7	65	1.9×10^3
	18	2.8×10^4	6.6×10^4

*Average results from 5 mice.



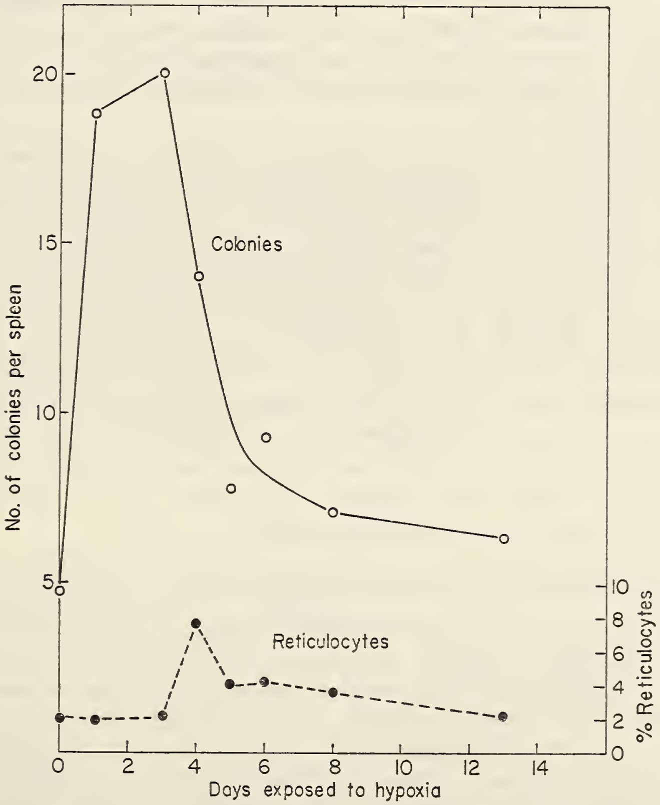
TEXT-FIGURE 3.—Virus titer in the plasma of normal and splenectomized mice. *Each point* is the result of pooled plasma from 3 mice. The virus in each sample was titered in 5 mice.

An examination of the virus titer in the plasma of normal and polycythemic mice, after RLV inoculation, has shown that polycythemia resulted in a reduced production of virus (table 3). The reduction in colony formation and virus production are presumably both due to a decrease in the number of target cells for the virus as a result of the suppressed erythropoiesis that occurs in polycythemia.

Removal of target cells in the spleen of normal mice by splenectomy, followed by intravenous inoculation of 6 CFU per mouse 8 days later, also resulted in a decrease in virus titer in the plasma (text-fig. 3).

Effect of hypoxia

In the experiments on the stimulation of erythropoiesis by hypoxia, mice were exposed to hypoxia for different times, injected with RLV, and then scored for the number of colonies 7 days later. The results show about a fourfold increase in the number of colonies per spleen after 1 day in hypoxia (text-fig. 4). This level of increase in number of colonies was



Text-figure 4.—Number of RLV-induced colonies per spleen, and percent of reticulocytes in the peripheral blood, at different times after exposure to hypoxia. Average results from 6 mice.

maintained after 3 days in hypoxia in this experiment, and after 2 days in other experiments, and then decreased. In agreement with the results of others (*8*), the increase in the percent of reticulocytes in the peripheral blood was observed after between 3 to 4 days in hypoxia. Similar results were obtained on exposure to hypoxia of either normal or polycythemic mice. As in normal mice, in the experiments with hypoxia and with polycythemia, the colonies in the spleen after RLV inoculation were colonies of erythroid cells.

DISCUSSION

The results on the types of leukemia induced by 14 cloned lines of RLV, produced by 3 successive inoculations of single colonies in the spleen, have shown that the progeny of a single CFU can produce two types of leukemia. The type of leukemia induced by RLV thus seems to depend on whether the normal target cells transformed into leukemia cells by the same virus are erythroid, myeloid, or lymphoid. The induction of different types of tumors after 3 successive single plaque isolations has been observed with polyoma virus (*9*), and evidence that the same virus can induce various types of neoplasms has also been obtained with avian myeloblastosis virus (*10*).

The experiments with polycythemia and hypoxia have indicated some properties of the target cells for the erythroid leukemia induced by RLV. A study of the erythroid cells in the spleen of polycythemic mice has indicated that in the suppressed erythropoiesis in polycythemia there are still some early erythroid cells classified as proerythroblasts, but no late erythroid cells classified as normoblasts (*11*). Since some RLV-induced colonies were obtained in polycythemic mice, this suggests that the proerythroblast can act as a target cell. The injection of erythropoietin into polycythemic mice produces an increase in the number of proerythroblasts even at 1 day after injection (*11*). The absence of an increase in the number of colonies in polycythemic mice, even at 14 days after virus injection, thus suggests that injection with RLV does not result in the production of a new supply of erythropoietin.

Further evidence that the erythroid target cells have already undergone some degree of differentiation was obtained from the experiments with the use of hypoxia as an erythropoietic stimulant. An increase in the number of colonies per spleen was obtained after 1 day in hypoxia, and the order of magnitude of this increase was about the same as the increase in the number of proerythroblasts obtained 1 day after injection of erythropoietin into polycythemic mice (*11*). The peak increase in the number of RLV-induced colonies was maintained up to 2–3 days in hypoxia, which has also been observed to be the time for a peak of increase in iron incorporation in spleen cells (*8*). In contrast to the increase of cells in an early stage of erythroid differentiation soon after exposure to hypoxia, no increase, and even a decrease after several days in hypoxia, has been observed in the number of stem cells as measured by their ability to form cell colonies in X-irradiated mice (*8*).

The above data thus indicate that the RLV target cells are not stem cells, but cells in an early stage of erythroid differentiation, such as proerythroblasts and possibly also erythroblasts. A comparison of the *in vitro* differentiation of RLV-induced cell colonies and normal cell colonies, produced in X-irradiated mice, could show to what extent the induction of RLV-induced erythroid cell proliferation is associated with a block that prevents full differentiation of the cells to mature erythrocytes.

SUMMARY

The Rauscher leukemia virus (RLV) was used to analyze the types of leukemia induced by cloned lines of virus, and to determine whether the target cells for the virus are stem cells or cells that have already undergone some degree of differentiation.

Fourteen cloned lines of RLV were produced by 3 successive isolations from single RLV-induced colonies in the spleen. Each line induced both erythroid and myeloid leukemia. These results indicate that 2 types of leukemia can be induced by the progeny of a single colony-forming unit.

The nature of the target cells for the erythroid leukemia induced by RLV was studied by the use of polycythemia as a suppressor of erythropoiesis, and of hypoxia as an erythropoietic stimulant. The suppression of erythropoiesis by polycythemia resulted in a decrease in the number of RLV-induced colonies in the spleen and a decrease in virus titer in the plasma. The stimulation of erythropoiesis by hypoxia resulted in an increase in the number of colonies in the spleen even after 1 day in hypoxia. The results indicate that the target cells for RLV are not stem cells, but cells in an early stage of erythroid differentiation.

REFERENCES

(*1*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*2*) Zeigel, R. F., and Rauscher, F. J.: Electron microscopic and bioassay studies on a murine leukemia virus (Rauscher). I. Effects of physicochemical treatments on the morphology and biological activity of the virus. J Nat Cancer Inst 32: 1277–1307, 1964.

(*3*) Siegler, R., and Rich, M. A.: Comparative pathogenesis of murine viral lymphoma. Cancer Res 24: 1406–1417, 1964.

(*4*) Pluznik, D. H., and Sachs, L.: Quantitation of a murine leukemia virus with a spleen colony assay. J Nat Cancer Inst 33: 535–546, 1964.

(*5*) Jacobson, L. D., Goldwasser, E., and Gurney, C. W.: Transfusion-induced polycythemia as a model for studying factors influencing erythropoiesis. *In*

Ciba Foundation Symposium on Haemopoiesis. London, Churchill Ltd., 1960, pp 423–445.

(*6*) Axelrad, A.A., and Steeves, R. A.: Assay for Friend leukemia virus. Rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology 24: 513–518, 1964.

(*7*) Jepson, J. H., and Lowenstein, L.: Effect of hypoxic guinea pig plasma upon erythropoiesis in the polycythemic mouse and polycythemic guinea pig. Acta Haemat (Basel) 31: 329–337, 1964.

(*8*) Bruce, W. R., and McCulloch, E. A.: The effect of erythropoietic stimulation on the hemopoietic colony-forming cells of mice. Blood 23: 216–232, 1963.

(*9*) Winocour, E., and Sachs, L.: Tumor induction by genetically homogeneous lines of polyoma virus. J Nat Cancer Inst 26: 737–753, 1961.

(*10*) Baluda, M. A., and Jamieson, P. P.: *In vivo* infectivity studies with avian myeloblastosis virus. Virology 14: 33–45, 1961.

(*11*) Filmanowicz, E., and Gurney, C. W.: Studies on erythropoiesis. XVI. Response to a single dose of erythropoietin in the polycythemic mouse. J Lab Clin Med 57: 65–72, 1961.

DISCUSSION

Dr. Law: It seems to me one of the significant questions which should be asked here is: What do you mean by erythroid and myeloid leukemias? Could you tell us in biologic terms or histopathologic terms some of the characteristics of erythroid and myeloid leukemias? For example, you used inbred strains of mice. Are they autonomous neoplasms?

Dr. Sachs: I am as you know not an expert pathologist and I would, therefore, prefer not to give a very detailed pathologic description. Dr. Resnitzky, the pathologist who diagnosed the leukemias, did not observe these types of leukemias in control animals of the same age as those inoculated with the virus. He will be publishing his results in detail elsewhere, and I can on my return supply you with his detailed pathologic diagnosis. Regarding the question of autonomy, since the leukemias contain virus, it was not possible to decide by transplantation into the same mouse strain whether they are autonomous.

Dr. Furth: Concerning Dr. Law's question: With Friend leukemia the cells at first are not autonomous. Whether you inject cells or cell-free virus you always find after inoculation a marked proliferation in the very same loci of "reticulum" cells. These mature into erythrogenic cells. The evidence that myeloid leukemia can be produced by Friend virus itself is new and requires further analysis. The situation, if true, is similar to what occurs in avian erythromyelosis, the virus of which, it has been long shown, can produce either or both diseases.

Dr. Block: There is a human counterpart to this mixture of erythroid and granulocytic leukemia, first described as erythroleukemia on the European continent and later in the United States. With this type of leukemia the patient presents with what looks like an erythroid leukemia—that is a marrow filled with erythroblasts. Subsequently, these cells disappear to be replaced by stem cells and immature granulocytes so the patient then dies of an acute granulocytic leukemia.

In the disease you're describing, is the double type of leukemia merely a result of the fact that the animals have died before one leukemia has replaced the other—in other words, that it really isn't two leukemias? Have you had a chance to follow these animals over a long enough period to determine whether this is a double leukemia or merely an erythroid hyperplasia which is then subsequently replaced by acute stem cell or acute granulocytic leukemia?

Dr. Sachs: Dr. Resnitzky found both types of leukemia in the same animals.

Dr. Barski: Can you make an analogy between your system of colony production in the spleen with that used currently in virology on cell monolayers under agar?

The situation may be different because under agar *in vitro* you can exclude secondary contamination, but in the spleen *in vivo* you have constant circulation. How can you exclude the erratic secondary contamination and transformation of cells by circulating virus produced by other colonies?

Dr. Sachs: As you know, a method for cloning murine leukemia virus *in vitro* under agar is not at present available. We tried to minimize possible contamination of the colonies, *a*) by isolating colonies at 8 days after virus inoculation, at which time no virus-induced colonies were obtained after injection of extracts from neighboring areas of the spleen with no visible colonies, and *b*) by 3 successive single colony isolations.

Dr. Stansly: I think I can make a useful comment on Dr. Law's question (whether myeloid leukemias are autonomous). We have been working with a virus leukemia the abnormal cells of which appear in the blood and elsewhere and are, we believe, myeloid. They differentiate in two directions—as erythroid cells and granulocytes. Many of these leukemias are transplantable. Therefore, in answer to Dr. Law's question, we can say that these leukemias are in that sense autonomous. The latency of our virus-induced disease is at least 3 months, whereas neoplasia develops in a matter of 1 or 2 weeks when the tumor is transplanted. There is rarely a problem, therefore, in distinguishing transplantation from virus induction of this leukemia, even though the transplanted cells may harbor virus.

Dr. Mirand: I want to confirm the fact that induced polycythemia can delay or even inhibit the appearance of Rauscher leukemia. Also, in our early work with Friend leukemia, we have noticed erythropoietin accelerated the erythropoietic phase of the disease (Proc. Amer. Assoc. Cancer Res. 4: 44, 1963; and Acad. Nazionale dei Lincei, Quaderno N. 65: 9–31, 1964).

Have you considered that one doesn't observe complete differentiation of the erythroid cell in the spleen because the erythroid cell being produced there is defective and is unable to mature under the influence of erythropoietin?

Dr. Sachs: We hope to test to what extent erythropoietin can overcome a possible block in differentiation induced by the virus.

Dr. Axelrad: I would like to present evidence that is in keeping with Leo's contention that the stem cell of Rauscher virus-induced leukemia is an erythroid precursor cell. Incidentally, for this I'll have to assume that Friend and Rauscher viruses, at least with respect to this leukemia-inducing function, are similar.

First, a study we did in which FF and ff mice were compared with respect to sensitivity to Friend virus showed that mutant (ff) mice differ from the wild type (FF) in that they have an anemia very early in life which becomes cured, but there is evidence from the work of Grüneberg and of Thompson, McCulloch, Siminovitch, and Till that the basic genetic lesion is retained through adult life. The lesion seems to be a defect in erythroid precursor cells which shows up when these cells are called upon to produce red blood cells rapidly.

We found roughly a tenfold decrease in susceptibility of these ff mice to Friend virus as compared to FF mice, which would be in keeping with the stem cell of the Friend virus-induced leukemia being an erythroid precursor, possibly an erythropoietin-sensitive cell.

Second, when we isolated individual spleen foci induced by Friend virus in susceptible mice, we found virus in them by injection of the focal cells into other susceptible animals. If we make suspensions of the focal cells and put these into serum containing radioiron (Fe^{59}), where the serum acts to provide transferrin, then we get uptake of the iron into about one third of the cells in a focus—that is, about a third of focal cells can incorporate iron. A substantial fraction of the iron incorporated into focal cells is in the form of heme.

We wanted to know whether the virus-producing cells and the iron-incorporating cells in the same focus were in any way related. A summer student, Mr. Lofsky, using radioautography, was able to look at *in vitro* incorporation of Fe^{55} into cells from spleen foci at the electron microscope level. He found that Fe^{55} radioautographic reactions could be localized in typical focal cells, with their large nuclei and nucleoli, masses of condensed chromatin, and large numbers of polyribosomes in the cytoplasm. These cells were evidently producing virus, as could be seen from the characteristic picture of budding at the surface of the cells and the presence of virus-like particles nearby in the intercellular spaces. This may mean that the virus-producing cells characteristically present in spleen foci induced by Friend virus can also be iron-incorporating cells, and is again consistent with the idea that the stem cell of Friend virus-induced leukemia is an erythroid precursor cell.

Dr. Dunn: We have been very much interested in the Rauscher virus. I have always been unhappy at its being so readily accepted as leukemia. We do not have such a disease process in normal mice or in any untreated mice. It is an interesting proliferative disease and I think worth any amount of study.

We recently found that blood transfusion will also inhibit the Rauscher reaction in mice, and propylthiouracil feeding will also depress it. It is also curious that some mouse strains fail to react to this virus.

I think the most interesting recent work was done by Dr. Swaen (J. Nat. Cancer Inst. 36: 1027–1048, 1966) of Holland when he was at the National Cancer Institute with Dr. Rauscher. He found that this virus injected into rats produced a thymic type of tumor. The virus was then recovered from the rats and has unquestionably produced lymphocytic leukemia in mice.

I appreciate very much Dr. Sach's paper. I think it is highly informative and may lead us somewhat closer toward a solution to this very complicated problem.

Tissue Culture and Serologic Studies of Mouse Leukemia Viruses[1]

Wallace P. Rowe, Janet W. Hartley, *and* Worth I. Capps, *Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland*

Our primary interest in mouse leukemia viruses lies in the area of their natural history—where do the viruses occur in nature, how do they spread, in what way does their presence become manifested as disease, how much genetic variation occurs under natural conditions, etc. A *sine qua non* for studies of this type is a sensitive system for detecting the viruses as they occur in nature, in which variants of differing pathogenicity will be equally well detected. Nearly as important for full understanding of the viruses are systems for quantitation of virus, for detection of antibody, for detection of antigens common to members of the group, and for detection of antigens specific for various members within the group.

To a considerable extent we have been able to attain or approach these objectives for the mouse leukemia viruses (*1*, *2*). The basic observations from which the rest of the studies proceeded were made by Dr. Huebner; he found that rats bearing transplants of a Rauscher virus-induced thymic lymphoma, supplied by Dr. Rauscher, frequently developed complement-fixing (CF) antibody to extracts of the tumor. Such sera also reacted with extracts of other virus-induced mouse and rat leukemias, spontaneous mouse leukemias, and certain radiation-induced mouse leukemias. A variable percentage of sera reacted with certain control tissues, particularly normal rat thymus, but by screening sera versus a battery of control and leukemic antigens, we can readily obtain serum pools which are specific for leukemic extracts. Our interpretation of this finding is that the serum is detecting a group-specific antigen shared by all mouse leukemia viruses. Thus, these serum pools regularly react with extracts of leukemias induced by the Gross, Rauscher, Moloney, Friend, WM1–B, PLL,

---

[1] Summary of paper presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

Kaplan, and Kirsten strains, and with spontaneous leukemias in AKR, C58, and C3H/Fg mice.

More important, these serum pools also react with tissue culture cells infected with most of these laboratory strains as well as with strains from natural sources. Using the development of CF antigen in tissue cultures as an endpoint, we can titrate, conduct neutralization tests, and isolate field strains. With Swiss (NIH strain) mouse embryo cultures we can regularly isolate virus from spontaneous leukemias (AKR and C58), from plasma of normal AKR, C58, and C3H/Fg mice, and by a less direct procedure, from AKR, C58, and C3H/Fg embryos. Consistent isolation of leukemia virus from such embryos requires growing their cells in culture for one or two passages; virus can then be recovered from the culture fluids by passage to the standard NIH mouse embryo system. One low leukemia mouse strain, C3H/HeN, has also yielded virus from embryos and plasma, but in a low percentage of mice, while two other low incidence strains, NIH and BALB/cN, have never yielded virus.

Development of optimal conditions for propagating mouse leukemia viruses in culture has in turn partially solved the problem of obtaining usable rat sera for the CF test. High titer tissue culture fluids generally induce CF antibody in rats, without problems of nonspecific reactivity.

Use of the tissue culture system for antigenic analysis of the various strains by neutralization tests is still in a preliminary stage. With sera of rats immunized with tissue culture virus, we find cross neutralization between the Rauscher, Moloney, and Friend strains, with the homologous titer the highest; potent antisera for members of this subgroup generally do not neutralize WM1–B, Gross, or an isolate from AKR leukemia.

Fluorescent antibody (FA) staining with the rat antisera has given results very comparable to the CF test. The reaction is group-specific, but tends to be stronger with the homologous strain. In all instances the FA-stainable antigen is restricted to the cytoplasm, appearing either as diffuse or finely granular staining. In preliminary studies, counting of fluorescent foci at 5 to 6 days after infection appears to offer promise as a quantitative tool. FA may also offer a method for rapid detection of field strains; a culture inoculated with AKR leukemia extract was positive in the FA test within 6 days after inoculation.

In preliminary surveys, use of tissue culture and tumor CF antigens for detection of naturally occurring antibody yielded completely negative results with mouse sera, but led to the surprising finding of CF antibody in a high proportion of normal rat colonies. The rat CF antibody is apparently a response to a member of the group less closely related to the mouse strains than they are to each other, since it neither neutralizes nor gives positive FA reaction with the mouse strains.

Another preliminary finding is interesting and promising. We have found that the sarcomagenic line of Moloney virus, which Dr. Moloney recently encountered (*3*), produces focal cytopathic lesions in mouse embryo cultures much like Rous virus (*4*). Also, focus formation is interfered with by prior infection with noncytopathic strains of virus. However, the interference system to date is far less sensitive than the antigen induction method for detecting virus, and thus is not yet a useful method for virus assay.

REFERENCES

(*1*) Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J.: Complement fixation and tissue culture assays for mouse leukemia viruses. Proc Nat Acad Sci USA 53: 931–938, 1965.

(*2*) Hartley, J. W., Capps, W. I., and Rowe, W. P.: Unpublished data.

(*3*) Moloney, J. B.: The application of studies in murine leukemia to the problems of human neoplasia. *In* Some Recent Developments in Comparative Medicine. Academic Press, London. In press.

(*4*) Hartley, J. W., and Rowe, W. P.: Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. Proc Nat Acad Sci USA 55: 780–786, 1966.

DISCUSSION

Dr. Sachs: Is the focus-forming sarcoma virus neutralized by non-focus-forming leukemia virus?

Dr. Rowe: It is inhibited in an interference test, and antiserum to the Friend and Moloney strains neutralized the virus, using focus formation as an endpoint.

Dr. Sinkovics: I would like to say a word about the multiplicity of the virus-neutralizing and cytotoxic antibodies, which is relevant to your statement. There is a group-specific antigen and a type-specific antigen in these viruses.

We work with two types of mouse sera. One is produced with killed virus and adoptive immunization. The mice produce both virus-neutralizing and cytotoxic antibodies.

In another type of mouse serum, which we produce with Moloney, Friend, Rauscher, or any other photodynamically inactivated leukemia virus, only virus-neutralizing antibodies appear and no cytotoxic antibodies, or only in a very low titer. The cytotoxic antibodies are quite nonspecific. There is tremendous cross-overlap between these three virus-induced leukemias in the cytotoxic test, and a definite but much lower degree of cross-reaction between these three viruses in the neutralization test.

Apparently, the cytotoxic antibody detects an antigen, which is perhaps the soluble antigen of these viruses, common for the group. On the other hand, the virus-neutralizing antibody detects another antigen which may be incorporated in the viral envelope and distinguishes the individual members within one group much better than the cytotoxic antibody.

Dr. Rowe: We do not actually have anything on the relation of the cytotoxic antibody to the antibodies we are detecting. George Klein has been testing a number of our sera for such toxicity, but I prefer to leave any conclusions for him, or at least to discuss them with him before we talk about it.

Dr. Tyndall: We have recently been able to obtain a cytopathogenic effect (CPE) and transformation associated with the Rauscher virus in a cell line developed by Dr. Wright of the Pfizer Company. We find this is dependent upon the medium employed, and I wonder whether you can affect lesions produced with your Moloney derivative by the medium you used? Can the CPE be repressed at all?

Dr. Rowe: We have not looked at it very much. We've only tried several different media, using horse serum, fetal calf serum, or regular calf serum as the base. Regular calf serum gives us the best CPE, but we may get better virus production in a medium with fetal calf serum.

Dr. Mirand: Dr. Tyndall, did you rule out pleuropneumonia-like organisms (PPLO) as an agent responsible for the CPE effect?

Dr. Tyndall: We have tried to culture PPLO from these cultures and have been unable to do it. We've also grown the cultures in the presence of 100 μg of kanamycin per ml of medium. This does not seem to affect the CPE or transformation, so as of now we have yet to rule in PPLO. Because you cannot culture PPLO—this is a very esoteric art—I wouldn't say definitely that it isn't there, but we haven't been able to detect it as yet.

Dr. Rowe: I would offer a word on the same caution about my own work. The tearing we see in tube cultures has made us suspicious that PPLO may be involved here, but likewise we have been unable to culture PPLO.

Dr. Sykes: We have been unable to confirm Dr. Tyndall's observations with V5. We have tried repeatedly to get CPE using 4 times concentration of vitamins and amino acids. We do get, from late passages, cell-free preparations of this culture producing active Rauscher disease in mice.

Dr. Wright: I don't mean to throw the book at Dr. Tyndall, but we tried to repeat his results once and much to our surprise we got just the opposite results. So I don't know exactly where the situation stands.

Dr. Trentin: With reference to the animals with primary virus-induced tumors that fail to develop antibodies—were these injected at birth and do you regard this as a manifestation of tolerance?

Dr. Rowe: They were injected at birth, but I wouldn't conclude anything about tolerance. The type disease they have is so different from that in the animals bearing a large transplanted tumor on their back, and I expect that they have a depressed immunologic response. Also, they just don't live long enough after development of large tumors to have time to get antibody response. A characteristic of these tumor-induced antibodies is that generally the longer the tumor persists at large size the better the antibody, and apparently lymphona just kills them too rapidly.

Dr. Trentin: There is a real need for a leukemia virus-free strain of mice or, failing that, of rats which might be used as foster mothers for mice to attempt to develop virus-free strains of mice. In view of the fact that all 7 germfree strains of mice tested by Dr. Morris Pollard developed either spontaneous leukemia or radiation-induced leukemia associated with electron microscopically evident virus particles, there is a real need for a "clean" strain of mice or rats. Have you applied these serological methods to try to develop such a strain? I notice that at least one of your germfree strains of rats showed no positives.

Dr. Rowe: We don't have the virus from the rats, so the antibody is the only indicator—an antibody to an unknown virus. The BALB/c and NIH mice look germfree or at least leukemia-virus-free from this one type of test, but somebody should look at them from the carcinogen-induced tumor point of view and see if the virus is present there.

Its absence in the embryo in quite susceptible cultures is apparently an important observation. They may have it, but it must be laterally spread if they do.

Dr. Salaman: I want to say a word, in connection with this paper, about an agent which Dr. Harvey isolated in our laboratory last year. I think it is similar to Dr. Moloney's sarcoma-producing agent. We shall hear more about this agent later on in the meetings, but the point relevant to this paper is that in the histology of the tumors—and, by the way, the tumors are diverse, several types are produced, not all sarcomas—one finds inflammatory as well as neoplastic areas and also areas of lymphoid cell necrosis, which as far as I could see from the pictures is analogous to what you were finding in tissue culture.

On the other hand, in a few preliminary trials in tissue culture, though the virus proliferated, we could see no foci. This was probably a technical failure.

With regard to serology, this also is in the preliminary stage, but there is no doubt in my mind that this virus also has a wide cross-reaction with other viruses.

Dr. Ioachim: I wanted to ask some details about the origin of the cultures supporting this replication of virus. Were special strains of mice used? Did you use cultures of specific organs or mixed embryo cultures?

Dr. Rowe: The cultures were minced whole embryo in secondary cultures. We've really only worked with two strains of mice—the BALB/c from the NIH colony and the randombred (or almost randombred) NIH strain. They both propagate the laboratory strains of leukemia virus, but the NIH strain cultures are far more sensitive for detection of field strains. It is certainly possible that there are more sensitive strains of mice than our NIH, but we just haven't looked in that direction.

Dr. Ioachim: Working with Gross leukemia virus, we used a rat embryonal thymus as compared with kidney and spleen from the same embryos and we observed a marked difference. The thymus replicated the initial virus in high concentrations after periods of up to 9 months as compared with spleen and kidney from the same embryos infected in the same way which did not replicate the virus. It seemed also that the cells were morphologically different.

Experiments on the Hematologic Diversification of Viral Mouse Leukemias[1]

A. Graffi, F. Fey, *and* T. Schramm, *German Academy of Sciences, Institute for Cancer Research, Berlin-Buch, Germany*

After continued studies on the same material for more than 12 years—a virus-induced mouse leukemia in the present instance—it is difficult to encounter entirely new findings in the field. Such has been the case with our mouse leukemia with which further work has consisted primarily in the investigation of questions raised earlier. The work described here concerns the study of certain recognized manifestations of heterogeneity of virus properties as indicated by differences in host response in this virus-induced mouse leukemia.

The first matter for discussion is that of the hematologic variations in the leukemias induced by our virus strain in the mouse, with respect, particularly, to the possible mechanisms responsible for this phenomenon.

During the first years of testing the leukemogenic activity of the virus strain isolated at our Institute, we observed that newborn mice infected by inoculation with virus-containing cell-free filtrates developed (*1*, *2*), almost exclusively, generalized myeloid leukemia with a high percentage of chloroleukemia. After serial cell-free transfers, primarily in the rat, and after passage of the virus in tissue culture, there were observed both a considerable increase in virulence, as judged by reduction of the latent period, and, also, a diversification of the hematologic characteristics of the disease (*3*, *4*). In addition to myeloid leukemias previously seen, reticulum cell leukemias and sporadic lymphatic and erythroblastic leukemias occurred in increasing proportions. In addition, we sometimes observed mixed types of leukemias, particularly in combination with malignant reticulum cells.

We were interested in determining the reasons and the mechanisms responsible for these differences in the hematologic response to infection with the same virus strain (*5*). Initially, we focused attention on the question of a possible relationship between the form of leukemia from

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

which we first obtained the virus-containing filtrate and the qualitative and quantitative changes to various other hematologic manifestations. To investigate this aspect of the problem, we prepared cell-free filtrates from hematologically different types of leukemia which occurred regularly after infection of large numbers of newborn mice with our virus strain. These filtrates were obtained in the usual way, with the enlarged lymph nodes and the thymus used as source material, and inoculated into mice of different inbred strains.

Filtrates used in this series of experiments were derived from the following types of leukemia: 1) mature myeloid, 2) immature myeloid, 3) paramyeloblastic, 4) erythroblastic, 5) reticulum cell, and 6) lymphatic leukemias.

Tables 1 through 6 show the results of the studies. Filtrates from *mature myeloid* leukemias (table 1) induced predominantly myeloid leukemia with a high percentage of chloroleukemia and differentiated myeloid forms. The injection of filtrates from *immature myeloid* (table 2) and from *paramyeloblastic* leukemias (table 3) led to an increased variation in the leukemia types. In addition to the myeloid type, a large number of leukemias of a different kind occurred, which consisted primarily of reticulum cell leukemia and, in some cases, lymphatic leukemia, with simultaneous reduction in the incidence of chloroleukemia and differentiated myeloid leukemia. In about 50% of the cases, filtrates from *erythroblastic* leukemias (table 4) produced the same disease picture, whereas administration of cell-free extracts from *reticulum cell* leukemias (table 5) induced the corresponding leukemia or a mixture of lymphatic or myeloid types, in addition to reticulum cells. Especially interesting were the results with filtrates from the *lymphatic* leukemias (table 6). This form of leukemia was rarely induced by our virus strain. With filtrates from myeloid and reticulum cell leukemias, the frequency of lymphatic leukemia was below 5%. However, if one of these rare lymphatic leukemias was used for filtrate preparation, then lymphatic leukemia was predominant and occurred in about 60% of the animals inoculated. In addition, reticulum cell leukemias occurred frequently, also mixed, in most cases, with lymphatic cells, whereas the myeloid and erythroblastic types were almost completely absent.

TABLE 1.—Filtrate prepared from mature myeloid leukemia

Type of leukemia	Cases	Percent	
Mature myeloid	22	30	∼60
Chloroleukemia	23	32	
Immature myeloid	12	17	
Paramyeloblastic	7	10	
Myeloid-reticulum cell	6	8	
Reticulum cell	2	3	
Lymphatic	0	0	

TABLE 2.—Filtrate prepared from immature myeloid leukemia

Type of leukemia	Cases	Percent
Mature myeloid	6	20
Chloroleukemia	4	12
Immature myeloid	6	20
Paramyeloblastic	5	15
Myeloid-reticulum cell	9	27
Reticulum cell	1	3
Lymphatic	1	3

TABLE 3.—Filtrate prepared from paramyeloblastic leukemia

Type of leukemia	Cases	Percent
Mature myeloid	5	12
Chloroleukemia	1	2. 5
Immature myeloid	10	23
Paramyeloblastic	13	30
Reticulum cell and mixed types of leukemia	13	30
Lymphatic	1	2. 5

TABLE 4.—Filtrates prepared from erythroblastic leukemia

Type of leukemia	Cases	Percent
Erythroblastic	6	52
	11	
Erythroblastic-myeloid	5	
Myeloid	6	29
Myeloid-reticulum cell	4	19
Reticulum cell	0	0
Lymphatic	0	0

TABLE 5.—Filtrate prepared from reticulum cell leukemia

Type of leukemia	Cases	Percent
Mature myeloid	7	22
Chloroleukemia	3	10
Immature myeloid and paramyeloblastic leukemia	4	13
Reticulum cell and mixed types of leukemia	17	55
Lymphatic	0	0

TABLE 6.—Filtrates prepared from lymphatic leukemia

Type of leukemia	Cases	Percent
Mature myeloid	4	5
Immature myeloid	2	2. 5
Reticulum cell	13	16
Reticulum cell-lymphatic	14	17
Lymphatic	48	59. 5

If these mature leukemia types, both the lymphatic and myeloid forms, are transferred in serial cell-free passages, the corresponding leukemia type is, at first, the prevalent form (table 7), but this is followed by a gradual diversification into other types.

These results were independent of the mouse strain used, since the experiments with three different strains (XVII, Agnes-Bluhm, and CBA) yielded identical findings. Apparently, the hematologic type of leukemia induced by the same virus strain is largely determined by the cytologic origin of the leukemia filtrate, *i.e.*, it depends on the type of cell in which the virus material used for infection was formed. This is particularly true of leukemia filtrates obtained from pure types of mature leukemias (myeloid, lymphatic, and erythroblastic types). The question was raised as to how such an influence might be explained. Based on electron microscopic studies of virus formation in the cell (*4*, *5*), one may postulate the following hypothesis: Virus reproduction proceeds, at least predominantly, by the process of budding at the cell surface. It should be remembered that, in addition to the constituents induced by the virus genome, portions of the outer membrane of the respective cell types may be incorporated into the enveloping membrane of the virus. It could be assumed, further, that the virus particles acquire a specific affinity for that cell type by which they were produced, resulting in a certain histotropism of the virus particles of the same virus strain corresponding to its immediate cellular origin (*3*, *6*), *i.e.*, filtrates obtained from lymphatic leukemia induce primarily lymphatic leukemia because the virus particles are usually picked up by immature lymphatic cells.

TABLE 7.—Diversification of a lymphatic leukemia within successive cell-free passages

Type of leukemia	First passage		Second passage		Third passage	
	Cases	Percent	Cases	Percent	Cases	Percent
Myeloid (differentiated)	—		1	~5. 9	2	~6. 7
Immature myeloid	—		—		1	~3. 3
Chloroleukemia	—		—		1	~3. 3
Paramyeloblastic	1	~2. 9	—		—	
Myeloid-reticular	2	~5. 8	2	~11. 8	5	16. 7
Reticular	—		1	~5. 9	3	10. 0
Reticular-lymphatic	6	17. 6	3	17. 6	5	16. 7
Lymphatic	25	73. 5	10	58. 8	13	43. 3

The connection between the type of leukemia and the cytologic origin of the virus material is further suggested by the following experiment: We prepared cell-free filtrates (1:100) from different organs of mice 20 days after infection of the newborns with our nonselected virus strain. In earlier experiments we were able to show that a few days after infection of newborns, the cell-free filtrates, prepared from different organs, exhibited a high virus content and a strong leukemogenic activity (table 8). The filtrates prepared from different organs showed pronounced differences in activity relative to the hematologic form of the leukemia

TABLE 8.—Organ filtrates from strain XVII mice 20 days after virus infection (dilution 1: 100)

Organ	Number of animals	Leukemia	%	Average of latent period (days)
Lymph nodes	30	25	83. 3	117. 6
Thymus	26	25	96. 2	146. 3
Spleen	38	33	86. 9	145. 1
Bone marrow	46	29	63. 1	137. 8
Liver	40	27	67. 5	167. 7
Heart	36	10	27. 8	139. 4
Brain	30	6	20. 0	158. 3
Pancreas	37	0	0	—

TABLE 9.—Organ filtrates from strain XVII mice 20 days after infection with the leukemia virus. Total 155 leukemias in 280 animals (dilution 1:100)

Type of leukemia	Lymph nodes	Thymus	Spleen	Bone marrow	Liver	Heart	Brain
				%			
Mature myeloid	4	0	9	7	4	30	∼33
Chloroleukemia	16	48	73	52	22	0	
Immature myeloid and paramyeloblastic	26	22	15	23	48	10	
Myeloid-reticular	30	17	3	7	4	40	∼67
Reticular	4	0	0	4		10	
Reticular-lymphatic	16	13	0	0	13	10	
Lymphatic	4	0	0	0	0	0	
Erythroblastic	0	0	0	7	8	0	

induced (table 9). That is, the use of filtrates prepared from spleen and bone marrow produced a high proportion (50 to 70%) of chloroleukemia. After inoculation of filtrates prepared from heart and lymph nodes, less than 16% of the cases were chloroleukemia. At the same time, the filtrates prepared from these tissues and also from liver and thymus induced a much higher degree of diversification of leukemia types, including lymphatic and reticulocellular types, than filtrates prepared from spleen and bone marrow, which caused almost exclusively myeloid leukemia. Erythroblastic leukemia developed rarely and was seen only after injection of bone marrow and liver filtrates. The latter results may be considered as preliminary observations and are presently under further investigation in repeated experiments. The data presented here are based on experiments with 280 survivors, of which 155 animals developed leukemia.

The second subject I should like to discuss is the *transfer of our leukemias by means of nucleic acid* (*RNA*) *from leukemic tissues.* Like Moloney (*6*) and Dalton and Moloney (*7*), we obtained a significant rate of occurrence of leukemia after injection of RNA from leukemic tissues into newborn mice (*4*, *8*, *9*). The incidence of 20% leukemia observed was low compared with that occurring after inoculation of virus-containing filtrates. However, the results of this experiment were rather convincing, when compared (*a*) with the findings with untreated controls and (*b*) with those of experiments in which the animals had been injected with ribonuclease-treated RNA. In the second instance, the leukemogenic activity of the material was abolished.

At about the same time, we began experimenting on cultivation of our leukemia virus in tissue culture. This work led to increasingly positive results in that virus multiplication *in vitro* could be demonstrated in different normal tissues (*4*, *10–12*) for several weeks and even after several passages (tables 10 and 11). The largest amounts of virus were obtained with mouse embryonic tissue and HeLa cells, whereas experiments with embryonic rat tissues yielded poor results, and practically negative results were obtained when calf and human kidney were used. We tried to use the technique for cultivation of the leukemia virus *in vitro* for the demonstration of an infectious leukemogenic nucleic acid. The nucleic acid isolated from leukemic tissue by means of phenol or dodecylsulfate was not directly administered to the animals, but primarily to cultures of mouse embryonic tissue and of spleen, thymus, or bone marrow of newborn mice. We thus intended to obtain a more sensitive method for the detection of infective nucleic acid. This would be true if leukemia viruses are produced after infecting the cells with RNA

TABLE 10.—Inoculation of nutrient fluid from leukemia-virus-infected tissue cultures into newborn mice

Tissue cultures	Number of animals / Number of leukemias	Leukemias (%)	Type of leukemia
I. Embryo of mouse			
a) Primary infected tissue culture	153/63	40. 5	Myeloid, 33 Reticular, 5 Lymphatic, 2 Myeloid and reticular, 6 Not determinable, 16
b) 1. Passage	28/15	53. 6	Myeloid, 5 Myeloid and reticular, 3 Not determinable, 7
II. Embryo of rat			
a) Primary infected tissue culture	55/20	36. 4	Myeloid, 10 Reticular, 4 Myeloid and reticular, 2 Not determinable, 4
b) 1. Passage	16/4	∼25	Myeloid and reticular, 4
III. Kidney of calf			
a) Primary infected tissue culture	40/3	∼7. 5	Myeloid, 1
b) 1. Passage	8/0		Myeloid and reticular, 2
IV. HeLa cells			
Primary infected tissue culture	16/9	∼56	Myeloid, 5 Myeloid and reticular, 2 Not determinable, 2

TABLE 11.—Relation between leukemogenic activity of the nutrient fluid and the time of cultivation

Time of cultivation, days after inoculation with virus	Number of changes of nutrient fluid	Animals/leukemias nutrient fluid from:		
		A Embryo of mice	B Embryo of rats	C HeLa cells
Primary infected tissue culture				
4	2	3/2	2/1	—
8	3	3/3	7/1	—
11	4	1/1	—	—
15	5	2/1	2/1	7/4
20	6	4/4	5/1	—
25	7	—	—	9/5
1. Passage				
11	5	2/2		
18	6	6/4		

in vitro. The infectivity of the nucleic acid, therefore, was tested so that both nutrient medium and cellular material, derived at different intervals (2–36 days) from the RNA-treated cultures, were injected into newborn mice. Table 12 shows that, particularly with RNA from leukemic tissue, a leukemia rate of about 10% was obtained, whereas DNA which still contained a certain amount of RNA, induced a lower incidence of leukemia, namely, 3%. Particularly striking was the result with RNA when the findings were compared with controls treated with material from the same cultures without nucleic acid treatment (0.6% leukemias). Thus, these results with a relatively large number of animals lend further support to the possibility of isolating infective nucleic acid from the leukemic tissue, though the expected increase in sensitivity could not be achieved by infecting tissue culture cells as alternate host.

Finally, I would like to present some preliminary results on the cell-free transmission, and thus the virus etiology, of chemically induced leukemias.

TABLE 12.—Occurrence of leukemia in mice inoculated as newborns with material from tissue cultures treated with nucleic acid

Treatment of culture		Number of animals inoculated with materials from tissue culture	Leukemias (%)
RNA	(+DNA)	425	38
	(phenol)		(9. 9)
DNA	(dodecylsulfate)	312	9
	(from DNP)		(2. 9)
Total nucleic acid	(dodecylsulfate)	86	4 (4. 6)
Untreated		169	1 (0. 6)

Five years ago I reported, together with W. Krischke, that cell-free filtrates from mouse leukemias developing after treatment of newborn or adult animals with 9,10-dimethyl-1,2-benzanthracene[2] generally did not show leukemogenic activity (*13*). The leukemia incidence was about 4%. To enrich virus material from leukemic tissues, a purification method as described by Moloney was used (*14*). The results obtained indicated the probability that some of these chemically induced leukemias contained a leukemia virus. Among 15 cases of chemically induced leukemia (table 13), the results with respect to the presence of virus were practically negative in 12 cases. However, the material from 3 leukemias produced by polycyclic hydrocarbons gave a leukemia rate of about 40%, presumably indicating the presence of a leukemia virus in these 3 cases. Especially, case 15 was significant, because not only was there a high leukemia rate, but there was a very short induction time. Our results were in accord with similar experiments by Toth (*15*) and Irino *et al.* (*16*).

The findings with chemically induced leukemias cannot be consistently interpreted as yet. The following three possibilities should be considered:

1) Like X-ray-induced leukemias, the chemically induced ones are possibly caused by a leukemia virus, as was demonstrated by Gross (*17*) and Lieberman and Kaplan (*18*). Generally, the absence of cell-free transmission of chemically induced leukemias may be due to inadequate methodological conditions.

2) In view of a certain spontaneous leukemia incidence in most mouse strains and a widespread latent infection in mice with leukemia viruses, it should always be expected that in animals treated with chemical car-

TABLE 13.—Experiments on the cell-free transmission of leukemias induced by carcinogenic polycyclic hydrocarbons (DMBA* and MCA†)

Leukemias induced by carcinogens	Number of mice surviving inoculation	Number of induced leukemias	Leukemias (%)	Time of induction (days)
Myeloid, 1	12	1	~8	329
Myeloid, 2	16	3	~19	274, 281, 288
Lymphatic, 3	16	0	—	—
Lymphatic, 4	6	0	—	—
Lymphatic, 5	9	2	~22	156, 104
Myeloid, 6	8	3	~37	57, 59, 242
Lymphatic, 7	23	1	~4	350
Lymphatic, 8	22	1	4	227
Lymphatic, 9	11	0	—	—
Lymphatic, 10	19	1	~5	664
Lymphatic, 11	4	0	—	—
Lymphatic, 12	15	0	—	—
Lymphatic, 13	7	0	—	—
Lymphatic, 14	12	2	~17	232, 533
Lymphatic, 15	6	4	66	53, 54, 55, 94
Total	186	18	~10	—

*DMBA = 7,12-dimethylbenz[*a*]anthracene.
†MCA = 3-methylcholanthrene.

---

[2] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

cinogens, single, so-called spontaneous virus leukemias may occur which naturally are transmissible by cell-free extracts. Consequently one might expect two etiologically different categories of leukemias in experiments of this kind, only one of which can be transferred by cell-free filtrates.

3) A leukemia virus which is latently present in the corresponding strain settles in the chemically induced leukemia as a passenger virus, in the same way as with numerous transplanted tumors of the mouse, *e.g.*, Ehrlich carcinoma, Sarcoma 37, Sarcoma I, etc., from which Friend, Moloney, and we have isolated our leukemia virus lines.

REFERENCES

(*1*) Graffi, A., Bielka, H., Fey, F., Scharsach, F., and Weiss, R.: Gehäuftes Auftreten von Leukämien nach Injektion von Sarkom-Filtraten. Naturwissenschaften 41: 503, 1954 and Wiener med Wschr 105: 61, 1955.

(*2*) Graffi, A.: Chloroleukemia of mice. Ann NY Acad Sci 68: 540, 1957.

(*3*) Fey, F., Gimmy, J., and Graffi, A.: Hämatologische und histologische Untersuchungen an Mäuseleukämien, die durch zellfreie Filtrate aus leukämischen Rattengeweben induziert wurden. Acta Biol Med German 3: 598, 1959.

(*4*) Graffi, A.: Neuere Untersuchungen über das Virus der myeloischen Leukämie der Maus. Progr Exp Tumor Res 1: 112, 1960.

(*5*) Fey, F., and Graffi, A.: Untersuchungen zur haematologischen Aufsplitterung der durch das Virus der myeloischen Leukämie induzierten Leukosen. Acta Haemat 33: 139, 1965.

(*6*) Moloney, J. B.: The recovery of infectious nucleic acid from a virus-induced lymphoid neoplasm. Acta Un Int Cancr 19: 250, 1963.

(*7*) Dalton, A. J., and Moloney, J. B.: Type C particles in tissues of mice with nucleic acid induced leukemia. *In* Electron Microscopy. Fifth Int Congr for Electron Microscopy, Philadelphia, Aug. 29–Sept. 5, 1962 (Breese, S. S., Jr., ed.). New York, Academic Press, Inc., 1962, vol 2, MM–7.

(*8*) Bielka, H., and Graffi, A.: Untersuchungen über die leukämogene Wirkung von Nukleinsäuren aus virusinduziertem Leukämiegewebe. Acta Biol Med German 3: 515, 1959.

(*9*) Bielka, H., Graffi, A., and Yen, C. Y.: Isolierung, Eigenschaften und leukämogene Wirkung von Nukleinsäuren aus virusinduzierten myeloischen Leukämien. Acta Biol Med German 10: 63, 1963.

(*10*) Graffi, A., Baumbach, L., Schramm, T., and Bierwolf, D.: Untersuchungen zur Frage der Züchtbarkeit des Virus der myeloischen Leukämie der Maus in der Gewebekultur. Z Krebsforsch 65: 385, 1963.

(*11*) Graffi, A., and Schramm, T.: Züchtung des Virus der myeloischen Leukämie der Maus auf homologer Grundlage in der Gewebekultur. Acta Biol Med German 11: 929, 1963.

(*12*) Schramm, T., and Graffi, A.: Züchtung des Virus der myeloischen Leukämie der Maus auf heterologen Grundlagen in der Gewebekultur. Acta Biol Med German 11: 934, 1963.

(*13*) Graffi, A., and Krischke, W.: Versuche zur Frage der zellfreien Übertragbarkeit chemisch induzierter Mäuseleukämien. Acta Biol Med German 5: 299, 1960.

(*14*) Moloney, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J Nat Cancer Inst 24: 933–951, 1960.

(*15*) Toth, B.: Development of malignant lymphomas by cell-free filtrates prepared from a chemically induced mouse lymphoma. Proc Soc Exp Biol Med 112: 873, 1963.

(*16*) Irino, S., Ota, Z., Sezaki, T., Suzaki, M., and Hiraki, K.: Cell-free transmission of 20-methylcholanthrene-induced RF-mouse leukemia and electron microscopic demonstration of virus particles in its leukemic tissues. Gann 54: 225, 1963.

(*17*) Gross, L.: Attempt to recover filtrable agent from x-ray induced leukemia. Acta Haemat 19: 353, 1958.

(*18*) Lieberman, M., and Kaplan, H. S.: Leukemogenic activity of filtrates from radiation-induced lymphoid tumors of mice. Science 130: 387, 1959.

DISCUSSION

Dr. Fiore-Donati: I would like to know what is the meaning of the term "paramyeloid" leukemia and, consequently, what is the difference between "paramyeloid" and "immature" leukemia. I would like also to know what is, according to the experience of Professor Graffi, the morphologic pattern of the leukemic forms exhibiting macroscopically the characteristics of chloroleukemia.

Dr. Graffi (translated by Dr. Kirsten): The difference between paramyeloblastic and immature leukemia as shown in the table is relatively minor. In the European hematologic literature paramyeloblasts are usually defined as primitive cells resembling myeloblasts with certain nuclear characteristics distinguishable from the ordinary stem cell.

The term "immature leukemia" is synonymous with what we would call stem cell leukemia.

Chloroleukemia cytologically can have both immature as well as mature cells of the granulocytic series. In other words, you can have myeloblasts as well as metamyelocytes. The diagnosis of chloroleukemia is based mostly on gross observations. These are localized tumors with a distinctive green color, just as is the case with human chloroleukemia.

Dr. Fiore-Donati: What type of histological changes, if any, has been observed in the thymus of mice bearing myeloid leukemia?

Dr. Graffi (translated by Dr. Kirsten): The diagnosis of the thymic tumors was made cytologically from the peripheral blood and from histological examinations of the thymus.

Dr. Brodsky: I certainly think we're all struck by the wide diversity of hematologic changes that occur with these mouse leukemias. I would like to record still another we've been seeing rather consistently with Friend leukemia and which is perhaps in our hands one of its most consistent manifestations, and that is the development of a thrombocytopenia. This occurs within about 48 hours after infection and frequently reaches its nadir in about 2 weeks after infection.

We've also noted that those animals dying of the disease early in infection—not necessarily dying of the so-called leukemic manifestations of the disease—frequently have a hemorrhagic diathesis associated with platelet counts of less than 25,000. The normal platelet count is about 800,000 to 1,000,000 per mm^3. It is also interesting that Friend virus particles were demonstrated in the cytoplasm of the megakaryocytes with electron microscopy.

Our other observation is that there has been a persistent anemia and not a polycythemia associated with this thrombocytopenia. Finally, on the basis of ferrokinetic and red-cell survival studies we feel that there is no evidence of ineffective erythropoiesis with this infection and that the altered erythropoiesis we're seeing is more or less a compensatory mechanism to a hemolytic anemia and not the type of alteration in ferrokinetics one generally associates with human erythroleukemia.

Dr. Mirand: We also see hematologic diversification in mice with Friend leukemia. We reported on this some time ago [Proc. Amer. Assoc. Cancer Res. 3 (#4) : 345, 1962; and Acad. Nazionale dei Lincei, Quaderno N. 65: 9–31, 1964].

Secondly, when you put Friend virus into newborn rats lymphoid leukemia is observed (Virology 17: 362–364, 1962). Your comment about an anemic response in Friend leukemia is correct; however, this depends on the strain of Friend virus one uses. I shall say something about this later.

We also see a reduction in platelet counts, particularly in Friend-infected mice given the strain of Friend virus that produces anemia.

Dr. Bianco: I have one more question concerning the different types of leukemia induced by extracts of leukemic thymic tissue. Did you say that from preparations of thymic tissue you had mainly lymphatic forms? If I remember correctly, the table showed 43% of myeloid and 18% of reticular forms and no cases of lymphatic leukemia.

Dr. Graffi (translated by Dr. Kirsten) : With the original virus isolate there was a diversification of the hematologic pattern induced by cell-free extracts prepared from the thymus, as was shown in the table. In other words, there were 43% myeloid leukemias. But with selective passage material apparently the pattern changed somewhat. More often lymphatic leukemias were reproduced with thymic preparations than were the other forms of leukemia.

Dr. Bianco: I would like to know what type of leukemia resulted from inoculation of viral RNA.

Dr. Graffi (translated by Dr. Kirsten) : Generally, the same pattern of various leukemic forms was observed but predominantly, as is the case with this virus, myeloid leukemias, chloroleukemias, and a few reticulum cell sarcomas.

Dr. Bianco: Was it protein-free when it was inoculated?

Dr. Graffi (translated by Dr. Kirsten) : That's a question of conscience. There is no such thing as an absolutely protein-free nucleic acid.

Descriptive Summary on Breast Tumors and Leukemia Induced in Female Wistar Rats Receiving Gastric Instillations of 3-Methylcholanthrene[1,2]

M. B. Shimkin, M. Acuff, D. Thatcher, *and* M. Gruenstein, *Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140*

DURING the period 1949–63, Harry Shay and his research group conducted a series of investigations on the appearance of mammary carcinoma (*1–8*), leukemia (*9–14*), and other neoplasms in female rats of the Wistar strain that received intragastric instillations of 3-methylcholanthrene.

From these investigations, as well as from subsequent or previously unpublished experiments, were selected control groups that had different amounts of carcinogen or different schedules of administration, but were maintained under similar conditions. Original data on individual rats were reexamined to provide information on the pharmacodynamics of this experimental carcinogenesis model, with the aim of providing insight into the possible mechanisms of action involved in carcinogenesis.

EXPERIMENTAL PROCEDURE

Female rats of the Wistar strain, raised in the Fels laboratories, were used in all experiments before 1963. More recently, Wistar rats were purchased from Carworth Farms, New City, New York. Comparative analysis showed that the responses of these animals were indistinguishable from the previous results.

When the rats reached 5 to 6 weeks of age and weighed between 60 and 80 g, they were placed in the appropriate experimental or control groups

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported, in part, by Public Health Service research grants CA-2590 and CA-07771 from the National Cancer Institute.

by random allocation. At this age and weight, the females approached sexual maturity as manifested by opening of the vagina.

The rats were housed singly in metal cages with wire-mesh bottoms, until 1963. Comparative studies subsequently performed, however, indicate no effect in regard to weight gain or carcinogenic response when several animals are maintained in one cage, with adequate food access and space. The rats were fed the Rockland rat/mouse diet and tap water *ad libitum.*

The carcinogen, 3-methylcholanthrene (MCA), was dissolved in olive oil, 0.5 cc for 2 and 5 mg, in 1.0 cc for 10 mg doses, and in 2 cc of warm oil for 100 mg doses. A rubber tube was passed into the stomach and the carcinogen introduced. This procedure was carried out daily, except Sundays and some holidays, for periods of up to a year in most of the groups reported. Some groups received single, weekly, or thrice weekly schedules, as indicated. The daily doses of carcinogen produced some variation in the total dose received by individual animals, depending on the day the animals entered the group and the number of Sundays and holidays during the carcinogen-administration period. The range of the total dose and the mean dose received by each group are given for each group.

The animals were examined several times a week, and once a week they were weighed and the subcutaneous tissues palpated for the presence of masses. The day of detection of grossly palpable tumors and their site were recorded on all individual animals. When the masses attained a gross diameter of approximately 1 cm, they were surgically excised under general ether anesthesia in experiments conducted before 1964. The rats were then returned to their respective groups and examinations for and surgical removal of additional tumors continued until the condition of the animal became poor or until the group was terminated, usually at 52 to 56 weeks after the initial dose of the carcinogen. In later experiments, the animals were observed for 4 to 6 weeks after the appearance of the first grossly diagnosed breast tumor, and then killed without previous excision of the tumor.

Animals killed were necropsied and relevant tissues appearing abnormal were prepared for histologic examination. Blood smears were obtained at necropsy in many, but not all, groups.

The number of animals per group was between 10 and 25, and larger numbers per dose usually indicate combinations of 2 or more different groups. At several dose levels, groups of animals were observed at various times during 2 to 7 years and yielded indication of reproducibility of the neoplastic reactions. With 2 mg given daily during 4 months, for a total dose of 204 to 214 mg, 5 groups of 17 to 37 animals observed between 1955 and 1962 had breast cancers in 53, 50, 70, 60, and 64 percent of the animals. With 10 mg daily for 1 month, for a total dose of 240 to 270 mg, 4 groups of 12 to 29 rats in each, surviving for 8 weeks, observed during 2 years had breast cancers in 61, 30, 33, and 50 percent, respectively.

Pathology

The histopathology of the tumors appearing in Wistar rats after gastric instillations of MCA was described in some detail in previous publications. The great proportion of breast cancers, fibroadenomas, and leukemias can be recognized correctly by gross examination, and in about half of the groups reported herein only questionable masses or tissues were examined histologically.

The most common tumors were glandular carcinomas of the breast, of different morphologic variants described previously (*1*, *15*). Fibroadenomas of the breast and lymphomas were also encountered, but much less frequently. Many of the animals with lymphoma had frank leukemia. The most frequent was the acute monocytoid or myeloblastic type, with many animals demonstrating green tissue infiltrates indicative of chloroma (*9*, *14*). Most animals with leukemia had splenomegaly and lymphadenopathy, and many also had hepatomegaly. Previous investigations (*10*, *12*) showed that these, as well as the lymphocytic leukemias, were transplantable.

In regard to carcinomas of the breast, metastases were found usually only in animals that were permitted to survive with large tumors for 12 or more weeks. As an example, among 32 rats killed 12 to 18 weeks after the appearance of one or more grossly detectable breast cancers, when the tumors had reached up to 6 cm in diameter, 5 rats had pulmonary metastases, 1 other rat had metastases to a lymph node, and another had metastases to a salivary gland. No investigation was made of bones, so that the occurrence of bone metastases was not recorded.

Nine rats had other malignant neoplasms. Among these were 3 cutaneous carcinomas, 2 subcutaneous sarcomas, 2 salivary gland tumors, and 1 sebaceous gland carcinoma; 1 animal had 3 pulmonary adenomas or adenocarcinomas. All occurred in rats that had received the carcinogen.

RESULTS

Table 1 presents the results in untreated rats and in 2 groups that received single gastric instillations of MCA, in doses of 10 and 15 mg. Tables 2, 3, and 4 record the results in rats that received daily instillations of 2, 5, and 10 mg MCA, respectively, for 5 days to 1 year. Table 5 summarizes more recent experiments in which thrice weekly doses of 10 mg and weekly instillations of 100 mg MCA were used.

Each table shows, by intervals of 4 to 8 weeks, the number of rats that were alive, the number that developed breast cancers, the number that were found to have other neoplasms, and the mean body weight. The total and the mean values are derived from data on individual animals.

Data are thus available on 912 rats, from 43 separate experimental groups. Of these, 54 were untreated and 858 received MCA in different doses and schedules.

Table 1.—Breast cancer and other neoplasms in female Wistar rats, untreated or receiving single intragastric instillations of 3-methylcholanthrene (MCA)—10 to 15 mg total

Group	MCA per dose (mg)	Period given	MCA total dose (mg)		Week of experiment								Breast cancer			Fibro-adenoma (No.)	Leukemia (No.)
					0	8	16	24	32	40	48	52+	Total Number	Total %	Mean time (weeks)		
1	0	—	0	Rats alive	54	54	54	54	52	52	51	30					
				Rats with breast cancer								1	1	2	50		
				Additional or other tumors									0			0	0
				Mean body weight	75	186	210	222	233	241	254						
2	10	Once	10	Rats alive	12	12	12	12	12	12	5	0					
				Rats with breast cancer							1		1	8	44		
				Additional or other tumors									0			0	0
				Mean body weight	67	178	209	222	235	240	259						
3	15	Once	15	Rats alive	23	23	22	22	21	21	21	16					
				Rats with breast cancer						1	1		2	9	40		
				Additional or other tumors					M*			2	2			0	1
				Mean body weight	76	178	204	217	225	231	242						

*Myelocytic leukemia.

Table 2.—Breast cancer and other neoplasms in female Wistar rats receiving intragastrically 2 mg 3-methylcholanthrene for 1 to 12 months, daily except Sunday

	Methylcholanthrene			Week of experiment												Breast cancers				
																Total tumors				
Group	Period given (months)	Total dose (mg)		0	8	12	16	20	24	28	32	36	40	48	52+	Number	%	Mean time (weeks)	Fibro-adenoma (No.)	Leukemia (No.)
4	1	52–54	Rats alive	36	34	34	34	33	32	31	29	29	28	25	23					
			Rats with breast cancer				3	1	1	2				2	1	10	28	28		
			Additional breast cancers				2	6			1	2		2		13				
			Rats with fibroadenoma or leukemia*									F		F					2	0
			Mean weight	64	171		202		219		233		240	249						
5	2	96–108	Rats alive	15	15	15	15	15	15	15	15	14	14	13	11					
			Rats with breast cancer			2	1							2		5	33	24		
			Additional breast cancers			1			1	1				2	1	6				
			Rats with fibroadenoma or leukemia											F					1	0
			Mean weight	66	155		185		199		213		220	226						
6	3	144–156	Rats alive	14	14	14	14	14	14	14	12	11	11	9	7					
			Rats with breast cancer			2	4		1	1		1		1		10	72	22		
			Additional breast cancers				2	2		1			2	1	1	9				
			Rats with fibroadenoma or leukemia																0	0
			Mean weight	67	175		198		210		218		228	222						

See footnote at end of table.

TABLE 2.—Breast cancer and other neoplasms in female Wistar rats receiving intragastrically 2 mg 3-methylcholanthrene for 1 to 12 months, daily except Sunday—Continued

	Methylcholanthrene			Week of experiment												Breast cancers				
																Total tumors				
Group	Period given (months)	Total dose (mg)		0	8	12	16	20	24	28	32	36	40	48	52+	Number	%	Mean time (weeks)	Fibro-adenoma (No.)	Leukemia (No.)
7	4	204–214	Rats alive	128	126	126	126	125	122	118	111	105	99	87	52					
			Rats with breast cancer		1	3	16	13	11	11	4	4	1	10	4	78	61	25		
			Additional breast cancers			1	5	20	12	21	17	10	17	11	15	129				
			Rats with fibroadenoma or leukemia							F			L	7F					8	1
			Mean weight	71	167		190		201		209		215	224						
8	5	262–264	Rats alive	18	18	17	17	17	17.	16	16	15	14	10	7					
			Rats with breast cancer					1	3	2		1		2	1	10	56	31		
			Additional breast cancers						1			1		1	2	5				
			Rats with fibroadenoma or leukemia											F	F				2	0
			Mean weight	67	161		187		195		207		213	213						
9	6	304–320	Rats alive	17	16	16	15	15	15	14	14	13	13	11	8					
			Rats with breast cancer				2	3	2	2	1	2		1		13	76	25		
			Additional breast cancers					1	1	3	4	2	3	4	2	20				
			Rats with fibroadenoma or leukemia										F		F				2	0
			Mean weight	65	162		184		198		214		203	225						

10	9	472–480	Rats alive	16	15	15	14	14	14	14	13	12	12	8	5					
			Rats with breast cancer			1	2	1	1	4	1		1	1		12	75	25		
			Additional breast cancers				2	1	2	2	7	2	4	5		25				
			Rats with fibroadenoma or leukemia									F		F					2	0
			Mean weight	66	155		183		200		206		205	208						
11	12	to 624	Rats alive	39	38	38	37	37	36	33	31	29	27	22	7					
			Rats with breast cancer			1	5	3	5	8	4	3	3			32	82	24		
			Additional breast cancers					8	5	6	10	11	6	20	2	68				
			Rats with fibroadenoma or leukemia							F				2F	F				4	0
			Mean weight	67	171		193		205		215		222	214						

*F, fibroadenoma; L, lymphocytic leukemia.

Table 3.—Breast cancer and other neoplasms in female Wistar rats receiving intragastrically 5 mg 3-methylcholanthrene for 5 days to 12 months, daily except Sundays

Group	Methylcholanthrene: Period given	Methylcholanthrene: Total dose (mg)		Week of experiment: 0	8	12	16	20	24	28	32	36	40	48	52+	Breast cancers: Total tumors: Number	%	Mean time (weeks)	Fibro-adenoma (No.)	Leukemia (No.)
			Rats alive	23	23	22	22	22	22	22	22	21	21	21	7					
12	5 days	25	Rats with breast cancer				1	1							1	3	13	29		
			Additional breast cancers					1							1	2				
			Rats with fibroadenoma or leukemia*											F					1	0
			Mean weight	71	170		208		221		234		241	250						
			Rats alive	46	46	46	46	46	44	44	44	43	41	39	33					
13	10 days	50	Rats with breast cancer			2		1		1			1	1	1	7	15	28		
			Additional breast cancers						1		1		1	1	1	5				
			Rats with fibroadenoma or leukemia											F	M, L				1	2
			Mean weight	70	168		202		215		227		235	249						
			Rats alive	22	22	21	21	20	20	19	18	18	18	17	5					
14	20 days	100	Rats with breast cancer			5	1	2	3					1	1	13	59	20		
			Additional breast cancers							3	1			7	3	14				
			Rats with fibroadenoma or leukemia																0	0

			Mean weight	71	165		201		219		229		237	245						
15	1 month	120–150	Rats alive	21	21	21	19	18	17	16	16	16	14	10	9					
			Rats with breast cancer			4	3	3		2						12	57	16		
			Additional breast cancers				1	1		3				3	2	10				
			Rats with fibroadenoma or leukemia								F			F					2	0
			Mean weight	69	157		184		202		218		227	238						
16	2 months	240–270	Rats alive	44	44	44	43	42	37	33	32	29	25	14	4					
			Rats with breast cancer			13	11	5	5	1	1			1	1	38	86	18		
			Additional breast cancers			10	38	22	16	16	6	9	4	5	1	127				
			Rats with fibroadenoma or leukemia						L		F			2F	2F				5	1
			Mean weight	69	165		191		207		216		217	224						
17	3 months	360–390	Rats alive	21	20	20	20	20	16	16	12	8	5	1	1					
			Rats with breast cancer			4	11	3		1		1				20	95	16		
			Additional breast cancers				27	24	29	18	5	2	5			111				
			Rats with fibroadenoma or leukemia										L						0	1
			Mean weight	74	161		188		205		208		-	—						

See footnote at end of table.

Table 3.—Breast cancer and other neoplasms in female Wistar rats receiving intragastrically 5 mg 3-methylcholanthrene for 5 days to 12 months, daily except Sundays—Continued

	Methylcholanthrene			Week of experiment												Breast cancers				
																Total tumors				
Group	Period given	Total dose (mg)		0	8	12	16	20	24	28	32	36	40	48	52+	Number	%	Mean time (weeks)	Fibro-adenoma (No.)	Leukemia (No.)
			Rats alive	21	21	21	20	17	16	9	8	4	3	1	1					
18	4 months	510-540	Rats with breast cancer			4	7	3	3					1		18	86	17		
			Additional breast cancers			2	13	15	41	17	6	6	1	3		104				
			Rats with fibroadenoma or leukemia							F, M									1	1
			Mean weight	66	142		168		179		191									
			Rats alive	21	21	21	18	17	15	13	12	9	8	5	5					
19	12 months	to 1520	Rats with breast cancer			3	5	5	2	3		1	1			20	95	20		
			Additional breast cancers			4	17	19	14	19	22	9	10	1	5	105				
			Rats with fibroadenoma or leukemia							F, M		M			F				2	2
			Mean weight	62	157		183		199		203		216							

*F, fibroadenoma; L, lymphocytic leukemia; M, myelocytic leukemia.

Table 4.—Breast cancer and other neoplasms in female Wistar rats receiving intragastric instillations of 10 mg 3-methylcholanthrene, for 1 to 4 months, daily except Sunday

	Methylcholanthrene			Week of experiment											Breast cancers				
															Total tumors				
Group	Period given (months)	Total dose (mg)		0	8	12	16	20	24	28	32	36	48	52+	Number	%	Mean appearance time (weeks)	Fibro-adenoma (No.)	Leukemia (No.)
20	1	240–270	Rats alive	87	73	70	67	65	63	61	55	46	29	17					
			Rats with breast cancer		1	5	9	11	2	1	1		2		32	37	18		
			Additional breast cancers			2	9	5	9	10	6	4	10	10	65				
			Rats with fibroadenoma or leukemia*			M		F,M				L		F				2	3
			Mean body weight	71	151		186		202		214		233						
21	2	480–540	Rats alive	59	49	39	37	33	33	29	27	22	14	3					
			Rats with breast cancer			5	10	2	2	3	2	1	2		27	46	20		
			Additional breast cancers				8	6	5	10	8	6	10	3	56				
			Rats with fibroadenoma or leukemia								M	M	F					1	2
			Mean body weight	71	134		178		195		209		211						
22	3	780–840	Rats alive	60	57	30	22	20	18	14	10	9	3	3					
			Rats with breast cancer			5	7	3	1	1	1				18	30	16		
			Additional breast cancers			2	11	11	10	12	5	7	6	3	67				
			Rats with fibroadenoma or leukemia					M	M		M, L	2M						0	6
			Mean body weight	73	127		172		185		214		—						

See footnote at end of table.

Table 4.—Breast cancer and other neoplasms in female Wistar rats receiving intragastic instillations of 10 mg 3-methylcholanthrene for 1 to 4 months, daily except Sunday—Continued

Group	Methylcholanthrene			Week of experiment											Breast cancers			Fibro-adenoma (No.)	Leukemia (No.)
	Period given (months)	Total dose (mg)		0	8	12	16	20	24	28	32	36	48	52+	Total tumors Number	Total tumors %	Mean appearance time (weeks)		
23	4	1020–1080	Rats alive	61	54	33	21	19	18	13	13	11	2	0					
			Rats with breast cancer			8	4	5	3		1	1			22	36	17		
			Additional breast cancers			7	6	13	21	18	7	3	10		85				
			Rats with fibroadenoma or leukemia			2M F	M	2M				M						1	6
			Mean body weight	73	137		160		200		194		—						

*F, fibroadenoma; L, lymphocytic leukemia; M, myelocytic leukemia.

Table 5.—Breast cancer and other neoplasms in female Wistar rats receiving intragastrically 10 mg 3-methylcholanthrene 3 times per week for 9 weeks and 100 mg in 1 to 3 weekly doses

Group	Methylcholanthrene				Week of experiment									Breast cancers		
	Per dose (mg)	Period given (weeks)	Total dose (mg)		0	8	12	16	20	24	28	32	36	Total tumors Number	Total tumors %	Mean appearance time (weeks)
24	10	9	270	Rats alive	24	20	19	10	2	2	1	0				
				Rats with breast cancer			14	4			1			19	79	12
				Additional or other tumors			12	22	8	1	2			45		
				Mean body weight	75	167		188		192						
25	100	1	100	Rats alive	10	8	8	8	8	7	7	7	6			
				Rats with breast cancer				1						1	10	16
				Additional or other tumors										0		
				Mean body weight	71	166		185		199		240				
26	100	2	200	Rats alive	10	8	8	8	7	6	6	6	6			
				Rats with breast cancer				1					1	2	20	27
				Additional or other tumors										0		
				Mean body weight	70	160		182		201		257				
27	100	3	300	Rats alive	10	8	8	8	6	5	3	3	3			
				Rats with breast cancer				2	1					3	30	19
				Additional or other tumors										0		
				Mean body weight	68	150		172		183		270				

Mortality and Body Weight

Table 6 summarizes the effect of MCA administrations on the survival of the animals. Mortality is slightly increased by daily doses of 2 or 5 mg, but is 18 percent by 8 weeks with daily doses of 10 mg. By 24 weeks the mortality includes not only the effect of MCA, but loss of animals from neoplasms, although many rats with tumors were maintained for up to 30 weeks following the appearance of breast carcinomas, which were surgically excised.

Table 6 also summarizes the effects of MCA on the gain in weight of the rats. When MCA is given in 1 to 20 doses, for a total dose of up to 100 mg, there is depression in weight gain of up to 5 percent by 8 weeks, which is practically recovered by 24 weeks. On continuous doses of 2 or 5 mg per day, there is a 10 percent weight depression at 8 weeks, which persists at 24 weeks at the same level. With 10 mg per day, for 1 to 4 months, there is a weight gain depression of approximately 25 percent, but among the surviving animals there is some recovery by 24 weeks, at which time they weighed approximately 10 percent less than the untreated controls.

TABLE 6.—Mortality and mean body weight of female Wistar rats receiving intragastric instillations of 3-methylcholanthrene (MCA)

			Rats alive at weeks of experiment					Body weight loss at	
	MCA dose		0 weeks	8 weeks		24 weeks		8 weeks	24 weeks
Groups	Schedule	Total	Number	Number	%	Number	%	% of untreated	
1	—	0	54	54	100	54	100	0	0
2–3	Once	10–15	35	35	100	34	97	4	1
4–11	2 mg/day	52–624	283	276	98	265	94	11	9
12–14	5 mg/day	25–100	91	91	100	86	95	10	2
15–19	5 mg/day	120–1520	128	127	99	101	79	16	10
20–30	10 mg/day	240–1020	267	233	87	132	49	26	12
24–27	30–100/wk	100–300	54	44	81	—	—	13	13

Proportion of Rats With Breast Cancer

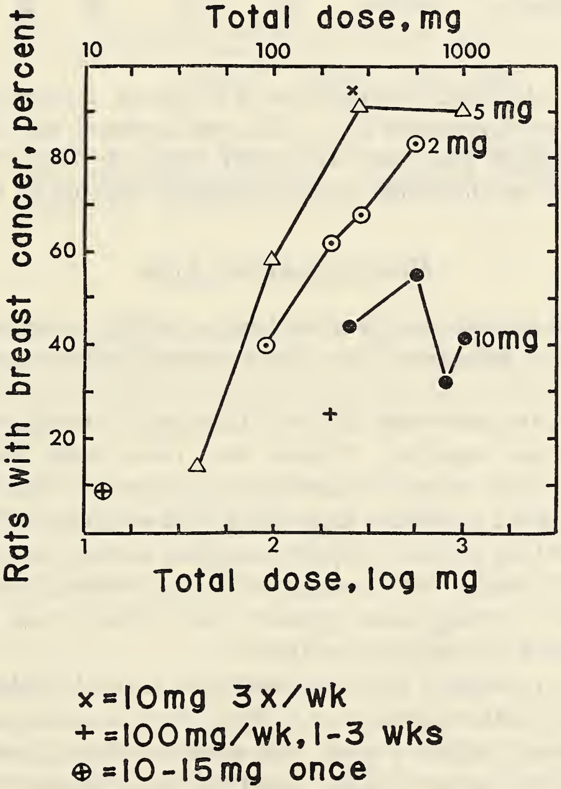
Among the 858 rats observed for up to 60 weeks, 429 developed one or more breast cancers. The relationships of dose schedule and total dose to the neoplastic response are indicated in table 7 and graphically presented in text-figure 1.

Of the 429 first tumors, only 2 were detected by palpation before 8 weeks; these 2 were recorded at 6 and 7 weeks, respectively. The number of rats alive at 8 weeks was thus taken as the "effective total," with adjustment in part for the higher mortality in groups receiving 10 mg MCA per day. From this gross analysis, it appears there is a direct relationship between total dose and the response with daily doses of 2 or 5 mg, with the latter being more effective. With 10 mg per day, there is a depression of response.

Actuarial analysis of the data, in which rats that died without mammary tumors between 8 and 52 weeks were assigned a proportional contribution to the animals at risk, showed that the responses to 5 mg daily doses were the highest but similar to those obtained with 2 mg schedules. The response to the 10 mg per day schedule retained some of the retardation effect observed in text-figure 1.

More recently, White *et al.* (*16*) analyzed these data, devising a technique in which individual animals were adjusted for varying exposure periods to obtain a "continuous exposure" function. There appeared to be a linear response between this function and time for groups receiving 2 or 5 mg. At the 10 mg level, however, an incidence-depressing factor was evident.

Recent experiments indicate that the proportional response to 10 mg given 3 times per week is equivalent to 5 mg given 6 times per week



TEXT-FIGURE 1.—Proportion of female Wistar rats developing breast cancer after intragastric instillations of 3-methylcholanthrene: 2, 5, and 10 mg doses given daily except Sundays and holidays. Data from table 7.

MURINE LEUKEMIA

TABLE 7.—Rats developing breast cancers: Summary of tables 1 through 5

		Total dose			Rats alive at 8 weeks	Breast cancer	
Groups	Schedule	Range	Mean (mg)	Log mean	(No.)	%	Mean appearance time (weeks)
1	—	—	—	—	54	2	50
2–3	Once	10–15	13	1. 11	35	9	42
4–6	2 mg/day	52–156	90	1. 95	63	40	25
7	"	204–214	210	2. 32	126	62	25
8–9	"	262–320	290	2. 46	34	68	28
10–11	"	472–624	565	2. 75	53	83	25
12–13	5 mg/day	25–50	40	1. 60	69	14	29
14–15	"	100–150	115	2. 06	43	58	19
16–17	"	240–390	290	2. 46	64	91	17
18–19	"	510–1520	1000	3. 00	42	90	19
20	10 mg/day	240–270	255	2. 41	73	44	18
21	"	480–540	510	2. 71	49	55	20
22	"	780–840	810	2. 91	57	32	16
23	"	1020–1080	1050	3. 00	54	41	17
24	10 mg 3×/wk	270	270	2. 43	20	95	12
25–27	100 mg/wk	100–300	200	2. 30	24	25	21

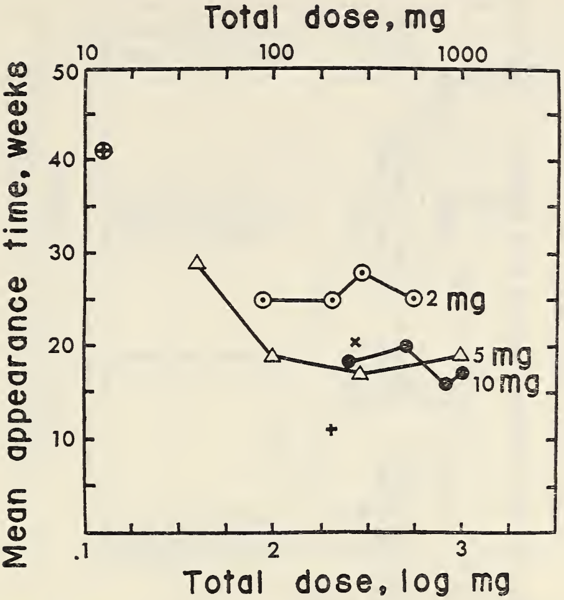
(group 24). However, the response is definitely inhibited by weekly doses of 100 mg (groups 25–27). Thus the increased mortality and depression of weight gain seen with daily doses of 10 mg or more are associated with an inhibition in the neoplastic response of the breast.

Mean Appearance Time

Table 7 also summarizes the mean time at which cancers of the breast were detected by palpation; these data are graphically presented in text-figure 2.

The results are somewhat different than to be anticipated from the frequency-response analysis. Tumors that occur after daily doses of 2 mg MCA appear at approximately the same mean time of 25 weeks. Tumors that occur following daily doses of 5 or 10 mg MCA, in total amounts of 100 mg or more, appear somewhat earlier, at a mean of approximately 18 weeks. At doses given in single administrations, or for a total of under 100 mg, there appears to be a clear inverse relationship between dose and mean appearance time.

It could be postulated that the carcinogen not only induces but also promotes the growth of the tumors. If this were so, the appearance time should be shorter, within a given dose schedule, when the carcinogen is administered for longer periods. Analysis does not show such a relationship. When MCA is given for a month in 2, 5, or 10 mg doses, the mean appearance times are 28, 16, and 18 weeks, respectively; when the administrations are continued for 4 months, the mean appearance times are practically identical, 25, 17, and 17 weeks, respectively.



TEXT-FIGURE 2.—Mean time of appearance of breast cancer in female Wistar rats after gastric instillations of 3-methylcholanthrene. Legends as in text-figure 1. Data from table 7.

Breast Cancers per Rat

The rat, with 12 breast trees, has the opportunity to develop breast cancer in not only each of the breasts, but in multiple foci of the same breast. Table 8 presents the multiplicity of tumors observed in female Wistar rats that were observed 4 to 30 weeks. Text-figure 3 indicates the dose relationships.

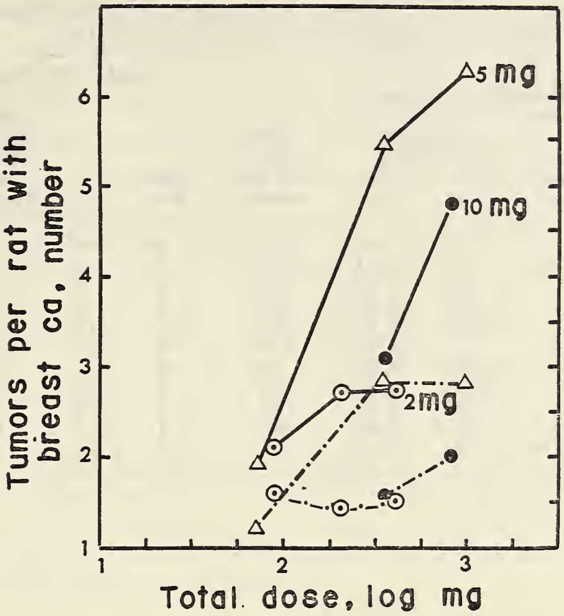
The number of tumors per rat is related to the total dose of MCA received, but is also influenced by the dose schedule. Thus the maximum numbers of tumors per rat are observed with the 5 mg daily dose, whereas with 2 or with 10 mg daily dose the mean number is approximately in the same range. The smaller number of tumors following the 10 mg schedule is undoubtedly another expression of the inhibition of mammary cancer development at levels of carcinogen toxic to the animal.

Data on multiple mammary tumors have been analyzed by Harris *et al.* (*4*), by considering the response per "breasts at risk." Unfortunately, not all breasts are equally liable to develop tumors, as indicated by the greater occurrence of tumors in the anterior breasts, and more than one tumor can develop from the same breast tree. This probably extends such more detailed considerations beyond the exactitude of the biological response.

MURINE LEUKEMIA

TABLE 8.—Number of breast cancers per rat with breast cancer by 4 and 16 weeks and total observation period after appearance of the first breast cancer

Groups	Methylcholanthrene			4 weeks		16 weeks		Total observation	
	Each dose (mg)	Total dose midpoint		Number of cancers/ rats with breast cancer	Mean tumors per rat	Number of cancers/ rats with breast cancer	Mean tumors per rat	Number of cancers/ rats with breast cancer	Mean tumors per rat
		mg	log						
1	0	—	—	1/1	1. 0	1/1	1. 0	1/1	1. 0
2–3	10–15	13	1. 11	3/3	1. 0	5/3	1. 7	5/3	1. 7
4–6	2	90	1. 95	34/22	1. 6	43/18	2. 4	53/25	2. 1
7	2	210	2. 32	101/71	1. 4	119/50	2. 4	207/78	2. 7
8–11	2	420	2. 62	95/63	1. 5	90/40	2. 3	185/67	2. 8
12–15	5	70	1. 85	36/30	1. 2	33/23	1. 4	66/35	1. 9
16–18	5	350	2. 54	193/69	2. 8	203/43	4. 7	418/76	5. 5
19	5	1000	3. 00	51/18	2. 8	23/6	3. 8	125/20	6. 3
20–21	10	360	2. 56	81/55	1. 5	101/41	2. 5	180/59	3. 1
22–23	10	930	2. 97	59/30	2. 0	73/16	4. 6	192/40	4. 8
24	10 mg 3/wk	270	2. 43	64/19	3. 4	—	—	—	—
25–27	100	200	2. 30	6/6	1. 0	—	—	—	—



TEXT-FIGURE 3.—Number of tumors per rat with breast cancer, at 4 weeks following appearance of the first breast tumor (*broken lines*) and at end of total period of observation (*solid lines*). Data from table 8.

Location of Breast Cancers

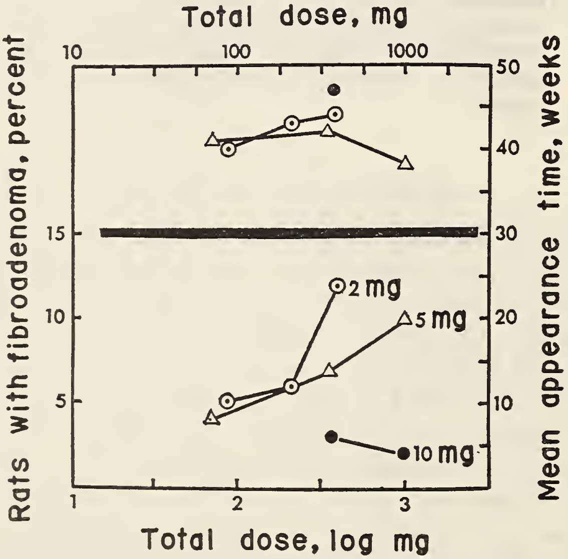
The location of the breast tumors in these rats was studied previously by Squartini (*17*), who found that the anterior thoracic-cervical breast glands were more frequently involved by tumor than the abdominal-inguinal glands. We repeated a limited determination and reiterated his findings. In 333 rats, the first breast tumor involved the anterior breast areas in 229 (70%) and the posterior breast areas in 104. The distribution between the right and the left side was almost identical. When all tumors in the animal were considered, the preponderance of anterior involvement was maintained at 67 percent of the tumors. There was no relation evident between the localization of breast cancers and the dose of MCA, the period of administration, or the appearance time of the tumors.

Fibroadenomas

Table 9 presents data on the appearance of fibroadenomas in 37 rats, and text-figure 4 is a graphic representation of the relationships. One or more rats developed fibroadenoma in each group of tables 2 through 4,

TABLE 9.—Appearance of fibroadenomas of the breast in female Wistar rats receiving intragastric instillations of 3-methylcholanthrene

	Methylcholanthrene				Rats with fibroadenoma			
	Each dose (mg)	Total dose midpoint		Rats alive at 12 weeks			Mean appearance time (weeks)	With breast cancer (No.)
Groups		mg	log		Number	%		
1	0	0	—	54	0	—	—	—
2–3	10–15	13	1. 11	35	0	—	—	—
4–6	2	90	1. 95	63	3	5	40	2
7	2	210	2. 32	126	8	6	43	5
8–11	2	420	2. 62	86	10	12	44	4
12–15	5	70	1. 85	110	4	4	41	1
16–18	5	350	2. 54	85	6	7	42	4
19	5	1000	3. 00	21	2	10	38	2
20–21	10	360	2. 56	109	3	3	47	3
22–23	10	930	2. 97	63	1	2	12	1
24–27	10–100	230	2. 36	43	0	—	—	—



TEXT-FIGURE 4.—Proportion of female Wistar rats developing fibroadenoma of the breast following intragastric instillations of 3-methylcholanthrene, and mean appearance time of fibroadenomas (*upper set* of data). Data from table 9.

in which MCA was given repeatedly and the survival of the animals prolonged by surgical removal of the breast cancers. Except for one rat that developed a fibroadenoma at 12 weeks, these tumors were first detected by palpation at 25 to 52 weeks of the experiments. However, the data of text-figure 4 are not affected by any appreciable degree if only rats alive at 24 weeks are considered in the determinations of the proportional responses.

The data indicate that with daily administrations of 2 or 5 mg for total doses of 70 to 350 mg fibroadenoma occurs in approximately 5 percent of the animals, which rises to 10 to 12 percent at doses of 420 to 1000 mg. There is definite depression of the response with daily doses of 10 mg, as seen with breast cancer. The mean appearance time is approximately the same at 40 weeks for all groups and dose levels.

Of the 37 rats with fibroadenoma, 22 also had breast cancers, usually removed before the appearance of fibroadenoma. This proportion of 60 percent is approximately the same as for the proportion of total rats with breast cancer and suggests that these two types of tumors are independent in the sense that their occurrence is neither inhibited nor promoted by the existence of the other.

Leukemias

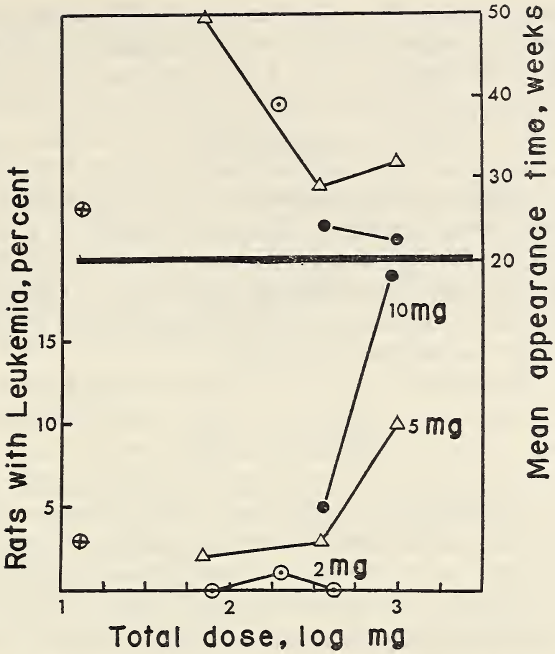
Table 10 summarizes the appearance of leukemia, and text-figure 5 expresses the data graphically. The relationships of responses to dose and schedule of MCA are quite distinct from those observed for breast cancer or fibroadenoma.

Daily doses of 2 mg MCA were ineffective in producing leukemia even at total doses of up to 400 mg. In this total dose range, 5 or 10 mg daily schedules produced leukemia in 3 to 5 percent, respectively. With 900 to 1000 mg total dose, 5 or 10 mg daily schedules produced leukemia in 10 to 19 percent of the animals. Thus there is a relationship both to daily and total dose of MCA. The mean latent period is inversely related to total dose.

Of the 26 rats with leukemia, 6 were diagnosed as having lymphocytic leukemia and 20 were diagnosed as myeloblastic leukemia; of the latter, 14 were chloroleukemias. The mean appearance time of the myeloblastic leukemias was 28 weeks, whereas of the 6 lymphocytic leukemias it was 36 weeks. Of the 26 rats with leukemia, 12 also had, or had had, cancer of the breast. It would appear that the two reactions are independent in that the occurrence of breast cancer did not influence the risk of leukemia or vice versa. However, as demonstrated by group 24, the detection of leukemia was possible because the animals were spared from termination with breast cancer by surgical removal of the mammary tumors.

TABLE 10.—Appearance of leukemias in female Wistar rats receiving intragastric instillations of 3-methylcholanthrene

Groups	Methylcholanthrene			Rats alive at 12 weeks	Rats with leukemia						With breast cancer (No.)
	Each dose (mg)	Total dose midpoint mg	Total dose midpoint log		Lymphocytic (No.)	Myeloblastic (No.)	Chloroleukemia (No.)	Total (No.)	%	Mean appearance time (weeks)	
1	0	0	—	54	0	0	0	0	—	—	—
2–3	10–15	13	1. 11	35	0	0	1	1	3	26	0
4–6	2	90	1. 95	63	0	0	0	0	—	—	—
7	2	210	2. 32	126	1	0	0	1	1	39	1
8–11	2	420	2. 62	86	0	0	0	0	—	—	—
12–15	5	70	1. 85	110	1	1	0	2	2	51	0
16–18	5	350	2. 54	85	2	1	0	3	4	29	2
19	5	1000	3. 00	21	0	1	1	2	10	32	1
20–21	10	360	2. 56	109	1	0	4	5	5	24	1
22–23	10	930	2. 97	63	1	3	8	12	19	22	7
24–27	10–100	230	2. 36	43	0	0	0	0	—	—	—
Totals, number					(6)	(6)	(14)	(26)			(12)
Mean appearance time, weeks					(36)	(28)					



TEXT-FIGURE 5.—Proportion of female Wistar rats developing leukemia following gastric instillations of 3-methylcholanthrene, and mean appearance time (*upper set* of data). Data from table 10.

DISCUSSION

The results summarized in this report represent an accumulation from experiments with Wistar rats and MCA as the carcinogen, performed during a decade by Harry Shay and his co-workers. The contributions of Huggins *et al.* (*18*, *19*) since 1959 show clearly that a more rapid and economical experimental model is available in the use of Sprague-Dawley rats and 7,12-dimethylbenz[*a*]anthracene (DMBA) as the carcinogen. We (*15*) have recently compared these two procedures, pointing out some of the differences as well as the similarities.

Following almost daily intragastric instillations of MCA, classed among the more potent carcinogens, in ranges up to and exceeding lethal doses, and with the known absorption and deposition of the carcinogen throughout the body tissues (*20*), one could have anticipated more neoplasms of a variety of organs and tissues. Yet for practical purposes only two types were induced: mammary tumors and leukemias. No neoplastic reactions were observed in the stomach or other portions of the gastrointestinal tract or the liver, such as are observed with 2-acetylaminofluorene and

2,7-acetylaminofluorene (*21*). The reaction to MCA, therefore, seems to be quite specific and quite limited from the standpoint of carcinogenesis.

In view of the intriguing work of Huggins and Grand (*22*), who reported that some subcutaneous sarcomas arose at the site of injection of presumably inert materials in rats fed MCA, it is also surprising that among our animals in which the mammary tumors were systematically removed by surgery, no sarcomas arose at the site of surgical procedures. The two subcutaneous sarcomas observed occurred at a site distant to surgery. This would suggest the trauma of surgery is not a carcinogenic stimulus in animals literally saturated with MCA. It also suggests that the presumably inert materials used by Huggins *et al.* (*22*) may be "cocarcinogenic" or "incomplete carcinogens."

Breast cancers induced in rats are known to be both hormone-dependent (*3*, *18*) and nutrition-dependent (*7*). They arise infrequently in males, and concurrent estrogen administration increases the frequency. In females ovariectomy or hypophysectomy reduces the development and causes regression of a considerable proportion of the grossly established neoplasms. Restriction of food sufficient to cause retardation of body weight gain by over 10 percent also retards the appearance and subsequent growth of the tumors (*23*, *24*). Recent data indicate that the latter effect can be dissociated from the hormonal effect, although obviously the effects are related.

To our knowledge, no systematic studies have explored the possible role of a viral entity in the carcinogen-induced breast cancers of rats. Foster-nursing of rats of several strains did not affect the response (*25*). Recently we have compared Wistar rats, descendants of a germfree colony, with the usual laboratory animals; the former, of course, had had a complete interruption of maternal milk and other postuterine contact. The frequency of mammary tumors following MCA was not affected, which further indicates that a milk-transmitted virus of the Bittner type is not demonstrable in rats. However, cell-free transmission and other more definitive techniques remain to be applied to the problem.[3]

In contrast with mammary carcinomas and fibroadenomas, leukemias induced with MCA appear to be neither hormone- or nutrition-dependent, at least to the same degree. Interestingly, the main type of leukemia observed in these animals was of the myeloblastic variety, with relatively few lymphocytic leukemias becoming manifest. This finding is reminiscent of the occurrence of acute myelocytic leukemia in human beings exposed to X radiation. A recent report by Weinstein and Moloney (*26*) indicates that particles compatible morphologically with other murine leukemias were seen in one of the transplantable leukemias initiated in this laboratory. Further investigations may thus lead to the conclusion reached by Kaplan (*27*), in his studies on leukemia in mice after X irradiation, that MCA exteriorizes in Wistar rats a latent virus involved in the genesis of the leukemias.

---

[3] Added in proof: *See* recent preliminary report, Scholler, J., Bergs, V. V., and Groupé, V.: Evidence for the cell-free transmission of chemically induced mammary carcinoma in the rat. Nature (London) 209: 1037, March 5, 1966.

SUMMARY

Data are presented on the appearance of mammary carcinoma and fibroadenoma, and leukemia among 912 female Wistar rats, 858 of which received single or repeated intragastric instillations of 3-methylcholanthrene, in 43 experiments conducted between 1954–64. The relationships between different amounts and different schedules of carcinogen administration, and the three types of neoplastic reaction, are described.

REFERENCES

(*1*) Shay, H., Aegerter, E. A., Gruenstein, M., and Komarov, S. A.: Development of adenocarcinoma of the breast in the Wistar rat following the gastric instillation of methylcholanthrene. J Nat Cancer Inst 10: 255–266, 1949.

(*2*) Shay, H., Harris, C., and Gruenstein, M.: Influence of sex hormones on the incidence and form of tumors produced in male or female rats by gastric instillation of methylcholanthrene. J Nat Cancer Inst 13: 307–331, 1952.

(*3*) Shay, H., Gruenstein, M., and Harris, C.: Reproducibility of method and hormonal influences in induction of breast cancer in rats by gastric instillation of methylcholanthrene. Acta Un Int Cancr 12: 733–741, 1956.

(*4*) Harris, C., Shay, H., Gruenstein, M., and Ciminera, J. L.: Breast tumor formation as a response to graded doses of methylcholanthrene and the effect of this drug on the pituitary glands of rats. Acta Un Int Cancr 15: 595–602, 1959.

(*5*) Shay, H., Harris, C., and Gruenstein, M.: Further studies in prevention of experimentally induced breast cancer in the rat. Some endocrine aspects. Acta Un Int Cancr 16: 225–232, 1960.

(*6*) Shay, H., Gruenstein, M., and Kessler, W. B.: Experimental mammary adenocarcinoma of rats: Some considerations of methylcholanthrene dosage and hormonal treatment. J Nat Cancer Inst 27: 503–513, 1961.

(*7*) Shay, H., Gruenstein, M., and Shimkin, M. B.: Effect of casein, lactalbumin, and ovalbumin on 3-methylcholanthrene-induced mammary carcinoma in rats. J Nat Cancer Inst 33: 243–253, 1964.

(*8*) Gruenstein, M., Shay, H., and Shimkin, M. B.: Lack of effect of norethynodrel (Enovid) on methylcholanthrene-induced mammary carcinogenesis in female rats. Cancer Res 24: 1656–1658, 1964.

(*9*) Shay, H., Gruenstein, M., Marx, H. E., and Glazer, L.: The development of lymphatic and myelogenous leukemia in Wistar rats following gastric instillation of methylcholanthrene. Cancer Res 11: 29–34, 1951.

(*10*) Shay, H., Gruenstein, M., and Glazer, L.: Uniform transfer to random bred rats of lymphatic leukemia induced by gastric instillation of methylcholanthrene. Proc Soc Exp Biol Med 75: 753–754, 1950.

(*11*) Shay, H., Gruenstein, M., Harris, C., and Glazer, L.: Transfer of myelogenous leukemia induced by gastric instillation of methylcholanthrene in Wistar rats. Blood 7: 613–622, 1952.

(*12*) Shay, H., Gruenstein, M., and Harris, C.: Experimental leukemias in the rat. Their development and transfer and some applications to the human problem. Acta Haemat (Basel) 14: 337–353, 1955.

(*13*) Shay, H., Harris, C., and Gruenstein, M.: The response of transplanted chloroleukemia in the rat to treatment with antileukemic drugs. Blood 11: 167–173, 1956.

(*14*) Harris, C., Burke, W. T., Gruenstein, M., and Shay, H.: The pathogenesis of transferred chloroleukemia in the Albino rat. Blood 8: 162–176, 1958.

(*15*) Gruenstein, M., Meranze, D. R., Thatcher, D., and Shimkin, M. B.: Carcinogenic effects of intragastric 3-methylcholanthrene and 7,12-dimethylbenz[*a*]-anthracene in Wistar and Sprague-Dawley rats. J Nat Cancer Inst 36: 483–502, 1966.

(*16*) White, R. F., Gruenstein, M., and Shimkin, M. B.: Tumor incidence in rats given repeated gastric instillations of 3-methylcholanthrene. Biometrics. In press.

(*17*) Squartini, F.: Fattori che influenzano la distribuzione dei tumori mammari indotti nel ratto. Lavori Ist Anat Univ Perugia 17: 281–289, 1957.

(*18*) Huggins, C., Brisiarelli, G., and Sutton, H., Jr.: Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors. J Exp Med 109: 25–42, 1959.

(*19*) Huggins, C., Grand, L. C., and Brillantes, F. P.: Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. Nature (London) 189: 204–207, 1961.

(*20*) Dao, T. L.: Level of 3-methylcholanthrene in mammary glands of rats after intragastric instillation of carcinogen. Proc Soc Exp Biol Med 102: 635–638, 1959.

(*21*) Morris, H. P., Wagner, B. P., Ray, F. E., Snell, K. C., and Stewart, H. L.: Comparative study of cancer and other lesions of rats fed *N,N′*-2,7-fluorenylenebisacetamide or *N*-2-fluorenylacetamide. Nat Cancer Inst Monogr 5: 1–53, 1961.

(*22*) Huggins, C., and Grand, L. C.: Sarcoma induced remotely in rats fed 3-methylcholanthrene. Cancer Res 23: 477–480, 1963.

(*23*) Davis, A. P., Gruenstein, M., and Shimkin, M. B.: Evaluation of chemotherapeutic agents employing mammary carcinoma induced by 3-methylcholanthrene in Wistar rats. Cancer Res, Part 2, 26: 1–18, 1966.

(*24*) Davis, A. P., Gropper, L., and Shimkin, M. B.: Response of 3-methylcholanthrene-induced breast cancer in rats to 31 chemotherapeutic agents. Cancer Res, Part 2, 26: 19–33, 1966.

(*25*) Huggins, C.: Induction of mammary cancer with polynuclear hydrocarbons. *In* Canadian Cancer Conf. New York, Academic Press Inc., vol 4, 1961, pp 81–88.

(*26*) Weinstein, R. S., and Moloney, W. C.: Virus-like particles associated with chloroleukemia in the rat. Proc Soc Exp Biol Med 118: 459–461, 1965.

(*27*) Kaplan, H. S.: Some implications of indirect induction mechanisms in carcinogenesis: A review. Cancer Res 19: 791–803, 1959.

DISCUSSION

Dr. Fiore-Donati: What is the incidence of leukemia in male rats?

Dr. Shimkin: We have induced leukemias in male rats, but our experience with them is rather sketchy since we were concerned mostly with mammary carcinogenesis. The impression is that males have approximately the same leukemia response as female rats.

Dr. Fiore-Donati: A comparison between males and females could give some information on the possible interference of mammary carcinoma with the incidence of leukemia in female rats.

Dr. Shimkin: We took care of this factor by removing the mammary carcinomas and continuing the observations. In the females we saw no association or interference between these neoplastic responses, as shown in the last table of our paper.

Dr. William Moloney: We have had a similar experience with the Shay as well as with several other forms of chloroma induced by methylcholanthrene and ionizing radiation. In our experience males and females seem equally susceptible. In the Wistar rat there seems to be a variety of viral particles, and we are having difficulty in proving that the virus-like particle we recently described is actually a leukemogenic agent. Since these particles were found in ascitic fluid from chloroleukemic rats, we are now attempting to create an ascites form of chloroma in our W/Fu rats. Since the male (Y) chromosome is distinctive in this inbred strain, it will be possible to establish, by cytogenetic methods, whether a cell-free passage of a leukemogenic agent has actually been accomplished. Using marker chromosomes, it may also be possible to determine if viral agents actually cause specific chromosome changes as proposed recently by Upton, Wald *et al.*

In studies on transplanted chloromas, we have observed in the Mound chloroma, carried now for 10 passages in our laboratory, consistent aneuploidy; instead of the normal $n=42$, most metaphases contain 78 to 81 chromosomes with 1 or 2 abnormal "marker" chromosomes. Another strain of this chloroma, transplanted at the Argonne Laboratory, had a hypodiploid line of 38 to 40 chromosomes with a consistent fragment present in each metaphase studied. In the Mound chloroma, the tumor was green and contained several enzymes; at the Argonne, the tumor lost its color and the enzyme activity had disappeared. Presumably, with an unstable hypodiploid stem line this tumor became more undifferentiated. I hope that during this conference someone will discuss the effect of viral agents on chromosomes.

Dr. Shimkin: Thank you very much, Dr. Moloney. Of course, you have gone quite a bit deeper into it than we have. What we have presented here is a retrospective analysis of a rather large experience. One of the things that struck us is that with higher doses of hydrocarbon we obtained a relatively high occurrence of myelocytic leukemia. This resembles the situation in Hiroshima and other human studies in which myelocytic leukemia is increased by exposure to ionizing radiation.

I am glad you also have the impression that males and females are equally susceptible to leukemia.

The only thing I can add to the virus phase is a demonstration that a Bittner-virus-type is not involved. We got hold of some descendants of germfree animals which, of course, had complete interruption from the milk of their original mothers, and we could see no difference in response either in terms of mammary carcinogenesis or of leukemia. Thus, at least the milk factor is not operative in these systems in the rat, or in the Wistar rat to be more specific.

We have not done any chromosome studies and since you're doing them we'll let you continue.

Some Experimental Evidence for the Influence of Genetic Factors on Viral Leukemogenesis [1, 2]

JUDITH R. TENNANT *and* GEORGE D. SNELL, *Sloan-Kettering Institute for Cancer Research, New York, New York, and The Jackson Laboratory, Bar Harbor, Maine*

THE general concept of genetically based host resistance or susceptibility to disease process has been well documented experimentally for infectious disease (*1, 2*) and virus infection specifically (*3, 4*), for tumorigenesis (*5, 6*) and for viral tumorigenesis in particular (*7–11*). Genetically homogeneous strains of animals are necessary to definitive studies of host response, and their development greatly stimulated research in this area. Early experiments in cancer research with defined strains of mice were concerned mainly with inheritance of susceptibility or resistance to *spontaneous* neoplasms and were summarized by Little in 1941 (*12*). Comprehensive reviews, including work in genetic analyses of host response to various carcinogens, have been done more recently by Law (*5*) and Heston (*6*). In most cases the conclusion has been that tumorigenesis is determined by multiple factors.

Except for two recent preliminary reports on leukemogenic viruses (*13, 14*), most investigations of genetic influence on mouse leukemia were done by crossing, backcrossing, and progeny-testing members of high and low leukemia strains, with careful tabulation of offspring for incidence of spontaneous leukemia as illustrated by the work of MacDowell, of Furth, and of Law (*15–17*). Although in the course of these studies two specific genes, *d* (dilution) (*15*) and *f* (flexed-tail) (*17*), were associated with expression of leukemia, analyses of ratios and observed differences in response among strains led to the conclusion that in leukemogenesis also tumorigenesis is not controlled by a single gene (*5, 16*). Evidence is given in this paper for the influence of a specific genetic locus on viral leukemogenesis, and, again, the data support the concept that multiple factors contribute to the total host response.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported by Public Health Service research grants CA-08087 and CA-01329 from the National Cancer Institute and by a Public Health Service Research Career Award K6-CA-21784.

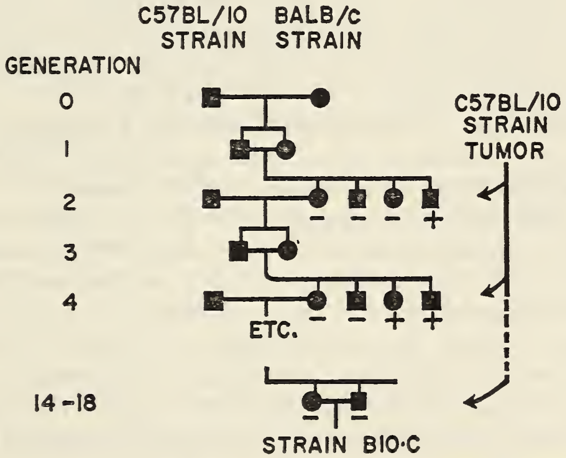
MATERIALS AND METHODS

Virus.—The history of the BALB/Tennant-leukemia (B/T-L) virus and techniques routinely used in preparation and injection of filtrates have been described in detail (*18*). Mice of the primary BALB/c strain, in which the original leukemia arose, succumb to lymphoid leukemia after an average latent period of 3 months, when inoculated intraperitoneally up to 5 days of age with virus through a 10^{-2} dilution (*19*). Inoculation at a later age, or with higher dilutions of virus, results in a lengthened latent period and a decrease in percentage of leukemias, as is to be expected from the data for other similar agents. The BALB/c strain is termed "primary" because it is the strain of virus origin and the strain in which the virus exerts its maximal leukemogenic effect. "Latent period" refers to the number of months elapsed between injection of the virus and leukemic death. In all cases reported here, unless otherwise specified, animals were inoculated at 1 to 4 days of age with B/T-L virus at passage 4 or 5 and were observed until death or for 12 to 18 months, with weekly palpation of each animal for evidence of enlargement of spleen or peripheral nodes. When the phrase "susceptibility (or resistance) to the virus" is used, it is understood that the only criterion of host response to the virus applied here is *leukemogenesis by* the virus. It is not yet known whether the virus is excluded at the cellular level in the resistant host, or at what stage the leukemogenic process is interrupted in those animals.

Mice.—In the present study 2 well-known inbred strains, BALB/cJ and C57BL/10ScSn, and 6 strains congenic with C57BL/10 were employed. Mice of the BALB/cJ strain were bred at the Sloan-Kettering laboratory from a small nucleus received from The Jackson Laboratory in 1963. All other mice used (C57BL/10 and its congenic lines) were bred at The Jackson Laboratory and shipped by air as pregnant females to the New York laboratory. For convenience in shipping, and to obtain a maximum number of litters within a minimum interval of time, advantage was taken, in the later groups of animals, of a recent study of factors which determine estrous synchrony in mice (*20*). On day zero, each group of prospective mothers was crowded 8 or 10 to a pen. On day 13 the females were mated individually to experienced males. Under these circumstances a substantial proportion of females mate on day 16. On day 31 shipment was made. Mice used in the earlier experiments, summarized in the text, were also produced at The Jackson Laboratory; for details, *see* (*14*, *19*).

Congenic strains are defined as strains essentially identical with a standard inbred strain except for a difference at a single locus. If the locus at which the strains differ is a histocompatibility locus, so that the strains mutually resist transplants of each other's tissues, the strains are referred to as *congenic resistant.* These strains can be produced by any one of three mating systems: the backcross system; the cross-intercross system; and the cross-backcross-intercross system (*21*). The cross-intercross system is illustrated in text-figure 1. In this system, mice in alternate generations following the initial cross are challenged with a transplantable tumor from one of the parental strains (C57BL/10 in the text-fig.) so that mice used in the next mating to this strain are of necessity resistant to its tissues. In the backcross system serotyping determines the resistant animals. Whatever the system, the final result is the extraction from the gene-complex of one strain (BALB/c in the text-fig.), and the introduction into the gene-complex of another (C57BL/10 in the text-fig.) of a histocompatibility gene and an accompanying chromosome segment. The chromosome segment is of indeterminate length, but will tend to be shorter as the number of matings to the background strain (C57BL/10) is increased. Other genes foreign to the background strain are present in the earlier generations, but are progressively eliminated with successive matings to the background strain.

The congenic resistant strains in these experiments were B10.A, B10.BR, B10.D2-new, B10.C(41N), B10.C(45N), and B10.C(47N). The first 3 strains differ from C57BL/10 at the histocompatibility-2 or *H-2* locus. The *H-2* allele in C57BL/10 is $H\text{-}2^b$, the *H-2* alleles in the other 3 strains are $H\text{-}2^a$, $H\text{-}2^k$, *and* $H\text{-}2^d$, respectively. The last 3 strains differ from C57BL/10 at the *H-1*, *H-9*, *and H-7* loci, the introduced locus always having come from BALB/c (abbreviated C). BALB/c is $H\text{-}2^d$, and strain B10.D2 resembles it in this respect. It is known that strain B10.C(41N)



TEXT-FIGURE 1.—The cross-intercross, or M, system of producing congenic resistant lines of mice. For example, following the initial cross of strain BALB/c to strain C57BL/10, intercrosses, crosses to C57BL/10, intercrosses, and so on, are repeated in successive generations. Offspring of each intercross are challenged with a C57BL/10 transplantable tumor, and only survivors (—) used to produce the next generation. In generation 14, 16, or 18, negative mice are mated to produce strain B10.C. [This diagram is taken from a previous publication (*21*), except that for the present purpose specific strains have been substituted for the "A" and "B" designations used previously.]

differs from C57BL/10 at the *c* (albino) and *Hb* (hemoglobin) loci as well as at *H-1*. All 3 loci are close together in the first linkage group. So far as is known, there are no differences between any of the other congenic strains and C57BL/10 except the specified histocompatibility difference, but in all probability some other undetected differences do exist. These are probably more numerous in lines B10.C(41N), B10.C(45N), and B10.C (47N), which were produced by 8, 9, and 8 matings, respectively, to C57BL/10, than in the other 3 congenic lines, each of which had 11 matings to C57BL/10. Strains B10.A and B10.BR were produced by Dr. Jack Stimpfling by the backcross system. The other congenic strains were produced either by the cross-intercross or the cross-backcross-intercross systems (*21*).

EXPERIMENTAL RESULTS AND DISCUSSION

The present series of experiments was begun in 1960, as a result of the observation that the host-strain range of a mouse leukemogenic virus just recovered (*18*) appeared to bear an almost inverse relationship to that of the Gross agent (*22*). The picture suggested a genetic basis for host response, and it was decided to investigate possible reasons for interstrain [and *intra* strain (*23*)] barriers to viral leukemogenesis in this system, and, more recently, reasons for *differences* in the spectrum of response among strains with each change in virus *source* (*19*, *22*, *24–28*). This last observation, that the virus-donor strain might be a factor in the host range of the virus, implies alteration in the virus in passage through the host; this is an interesting hypothesis and will be discussed later.

The Gross Passage A virus was recovered from a lymphoid leukemia of the AKR strain, a member of the histocompatibility-2^k ($H\text{-}2^k$) group of strains, and exerted its major leukemogenic effect in members of that group. The B/T-L virus used in the present studies, on the other hand, was recovered from a lymphoid leukemia arising in the BALB/c strain ($H\text{-}2^d$), and was leukemogenic for neither of two $H\text{-}2^k$ strains tested at that time. Because of these results, the first approach made to the problem of interstrain resistance barriers was an investigation of possible relationships between the histocompatibility-gene constitution of prospective hosts for the B/T-L virus and their susceptibility to leukemogenesis by the virus. For purposes of clarity, virus-host-range data previously reported (*14*, *19*) are briefly summarized.

Summary of Results Previously Obtained

Inbred strains.—A spectrum of 11 strains of mice challenged with undiluted virus (10% leukemic tissue filtrate) responded in one of three distinct ways: highly susceptible (93–100%), highly resistant (0–25%), or intermediate (68–80%) (*19*). The most pronounced differences in

TABLE 1.—Effect of hybridization with the primary (BALB) susceptible strain on viral leukemogenesis in each of two resistant strains of mice*

Resistant strain	Leuk. mice/total mice (% leuk.), latent period, months		
	Inbred	F_1	F_2
C3HeB/FeJ	3/69 (4) 12.0	23/25 (92) 4.8	20/26 (73) 5.3
C57BR/cdJ	2/22 (9) 14.5	0/15 (0) —	9/23 (39) 3.2

*Mice were inoculated at 1 to 4 days of age with undiluted virus and observed until death or from 10 to 17 months. Table summarizes data previously published (*14*).

response, when both percentage and latent period are considered, were those between the primary BALB/c strain and the 4 resistant *H-2*^{k} strains (C3HeB/FeJ, C57BR/cdJ, AKR/J, and C58/J). However, inconsistencies in the data were immediately evident from the point of view of an all-or-none *H-2* hypothesis, and it was evident that the *H-2* locus could not be *alone* responsible for susceptibility or resistance to leukemogenesis by the virus. But multiple influences on the leukemogenic process were to be expected, and the objective was to determine to what *degree* a given factor could influence expression.

F_1 generations.—F_1 generations of crosses between BALB/c females and males of each of 8 other strains were then tested for susceptibility to the same concentration of virus, to determine whether there was a consistent pattern of dominance for either extreme of response. In this generation, susceptibility persisted, or overrode resistance in all cases, except for the C57BR cross, in which the hybrids expressed the total resistance of the resistant parent (*14*). Because of this striking difference in response, C57BR/cd and a second *H-2*^{k} strain, C3HeB/FeJ, were chosen for further comparative study. Both strains have a negligible incidence of spontaneous leukemia.

F_2 generations.—Segregation of factors in the second hybrid generation appeared to confirm roughly a dominance for susceptibility in the C3H cross and for resistance among the C57BR hybrids (*14*) (table 1). Individual animals of the F_2 generations, serotyped for the major antigenic specificities controlled by the *H-2 d* and *k* alleles, showed no consistent correlation between *H-2* isoantigenic type and subsequent expression of leukemia. If the response of these two hybrid groups is compared, it is obvious that each of the resistant strains (both *H-2*^{k}) brought to the cross a qualitatively or quantitatively different complement of genetic information relative to leukemogenesis by the virus.

Results of Current Experiments

The availability of congenic resistant strains of mice, developed at The Jackson Laboratory for use in the identification and mapping of histocompatibility loci, offered a definitive, ready-made tool for analysis of the role played in viral leukemogenesis by histocompatibility factors. The family of strains chosen shares in common the genetic background of C57BL/10ScSn, except for a chromosomal region specifically associated with one or another of the 11 histocompatibility loci (or alleles thereof) so far identified (*21*). (*See* text-fig. 1 and Materials and Methods.)

The influence of the H-2 locus.—Preliminary data were presented in an earlier paper (*14*) for the first congenic pair differing at the *H-2 locus.* B10.D2 ($H\text{-}2^d$) showed a significant increase in susceptibility to the virus over its congenic partner strain, C57BL/10 ($H\text{-}2^b$), which was of intermediate susceptibility as compared to the full susceptibility of the primary reference strain, BALB/c. This pair was of particular interest since *d* is the allele carried at the *H-2* locus by BALB/c, the strain primary to the virus. The studies using congenic lines differing at the *H-2* locus have since been expanded to include 2 other strains, B10.A ($H\text{-}2^a$) and B10.BR ($H\text{-}2^k$). The data for all 3 of these congenic strains, plus the 2 reference strains, are summarized in table 2. In these later experiments, all mice were challenged at 10^{-2} as well as at 10^{-1} dilutions of virus, on the theory that, at greater dilution, response differences should be magnified. The increased susceptibility, noted earlier, among mice bearing the $H\text{-}2^d$ allele was confined. The *b* allele of C57BL conferred a higher degree of resistance than any other allele tested. Lilly *et al.* (*13*), using the Gross virus, found that, among serotyped F_2 individuals, the presence of the $H\text{-}2^b$ allele in homozygous state or heterozygous combination correlated well with resistance to that virus. Also in this connection, it is interesting that the C57BL strain is generally regarded as a low tumor line (*12*), as comparatively resistant to other oncogenic viruses (*25*, *27*, *28*), and as an "immunologic reactor" (*29*, *30*). Apparently the $H\text{-}2^k$ allele was not responsible, except perhaps in a minor degree, for the resistance in the strains of that group originally tested (*19*). That it may be partially responsible is indicated by the relative resistance of lines B10.BR ($H\text{-}2^k$) and B10.D2 ($H\text{-}2^d$); the line with the *k* allele showed a 10% lower incidence of leukemia.

TABLE 2.—Comparative effects of four *H-2* alleles upon leukemogenesis by the B/T-L virus

Strain*	*H-2* allele	Leuk. mice/total mice (% leuk.), latent period, months					
		10^{-1}			10^{-2}		
BALB/c	*d*	25/25	(100)	3. 1	67/67	(100)	2. 8
C57BL/10	*b*	32/52	(62)	6. 5	20/73	(27)	6. 8
B10.D2 (new)	*d*	50/53	(94)	3. 6	21/42	(50)	4. 3
B10.A	*a*	23/26	(88)	3. 2	31/55	(56)	3. 7
B10.BR	*k*	21/25	(84)	4. 3	24/56	(43)	4. 9†

*Each of the congenic strains listed differs from the C57BL/10ScSn strain by one *H-2* allele, but all share common identity with that strain at all other histocompatibility loci. BALB/c was the strain of virus origin.

†Animals listed are over 1 year of age, except for 39 of this group, which were tabulated at 6 months.

TABLE 3.—Comparative effects of *non-H-2* loci on leukemogenesis by the B/T-L virus

Strain*	*H*-locus (allele)	Leuk. mice/total mice (% leuk.), latent period, months					
		10^{-1}			10^{-2}		
C57BL/10	$H\text{-}1^{c}$, $H\text{-}7^{a}$, $H\text{-}9^{a}$	32/52	(62)	6. 5	20/73	(27)	6. 8
B10.C (41N)	$H\text{-}1^{b}$	28/41	(68)	5. 5	4/22	(18)	6. 2
B10.C (47N)	$H\text{-}7^{b}$	34/56	(61)	5. 4	5/24	(20)	6. 0
B10.C (45N)	$H\text{-}9^{b}$	27/37	(73)	5. 3	7/36	(19)	7. 0†

*Each of the congenic partners differs from the C57BL/ScSn strain by a difference at one histocompatibility locus, so that all the strains listed share the *H-2* and all other loci not specified.

†Twenty-five mice in this group were 6 months of age at time of tabulation; all others listed were over 12 months of age.

The over-all results of substitution of alleles at the *H-2* locus confirm a genetic basis for susceptibility or resistance to viral leukemogenesis; that is, replacement of a specific region of the IXth linkage group, by any of three alternative homologous portions, has resulted in a direct and significant change in host response to the virus. Identification of the specific functional unit responsible is less obvious. Because of the one consistent difference at the *H-2* locus, there is some presumption that the observed differences in susceptibility to the virus are due to the difference at that locus. However, as previously mentioned, a role for other introduced loci cannot be ruled out in the use of congenic lines.

The influence of non-H-2 loci.—Three congenic strains differing from C57BL/10 at *non-H-2* loci were then tested for susceptibility (table 3). Results were negative at the higher dilution of virus, but at 10^{-1} 2 of the strains [B10.C (41N) and B10.C (45N)], representative of the *H-1* and *H-9* loci, respectively, were slightly more susceptible (6–11%) to induction than the C57BL/10 partner, at an average latent period of 1 month less.

The source of the histocompatibility gene for all *non-H-2* lines was strain BALB/c. Further, if an essentially additive effect on susceptibility to leukemogenesis of substitution of the *H-1*, *H-2*, and *H-9* alleles of BALB/c for those of C57BL/10 can be assumed, then in theory the susceptibility of a hypothetical animal with such a triple substitution, when inoculated with the virus at a 10^{-1} dilution, would approach the 100% susceptibility of the primary strain. However, the data for the 10^{-2} virus dilution in these *H-1*, *H-2*, and *H-9* lines indicate a considerable resistance barrier in C57BL/10 still unaccounted for.

GENERAL DISCUSSION

The working hypothesis in these investigations has been that the leukemia virus particles, having become coated with cell membrane of a donor host in the process of their maturation and release from those cells, are therefore unacceptable to histo*in*compatible future hosts. In that case, the "strength" of the histocompatibility barrier between the two hosts and the dosage of virus would be critical to initiation of infection. The present data do not dispute this hypothesis. Several pieces of circumstantial evidence link histocompatibility relationships and host susceptibility to virus disease. For instance, it is established that most of the specific isoantigenic material in animal cells is recoverable from the membranous portions (*31–33*). This is exactly the material of which the outer membrane of several of the RNA viruses is known to be partially constituted, when the virus is completed at the cell membrane, as has been demonstrated for some "infectious" (*34–36*) and tumor viruses, including mouse leukemia virus (*36–39*). Pertinent here also might be a reference to the recent controversy concerning disease susceptibilities of human populations, as related to demonstrated antigenic similarities between the isoantigens of the human A-B system, and antigens of certain infectious viruses (*4*, *40*). So, perhaps sometimes it is more than coincidental that there be an association between histocompatibility factors of the prospective host and resistance to viruses coated with similar antigens from a previous donor host. What could in theory be more critical to host-virus interaction, at the cellular as well as at the systemic level, than the quality of their two surfaces? It becomes plausible, for instance, to suppose that some viruses would not have the "key to unlock" membranes of some histocompatibility structures. In this connection, it should be remembered that histocompatibility antigens are chemical entities quite apart from their antigenicity, and that their effect upon virus particles (host-coated or not), coming in contact with them at a cell surface, may be quite unrelated to their antigenic potential.

However, the specific site of action of the *H-2* locus is not known, and the proposed coating of virus particles need not be the only means through which the *H*-genes might affect host reactivity. Transduction of genetic material, *e. g.*, an *H*-locus, by the virus from one host to the next cannot be ruled out as a mechanism of action, although this seems a more likely mode of action for a DNA virus where replication of the virus apparently takes place in the cell nucleus. One wonders what association there might be between such considerations and the appearance in virus-induced tumor tissues (including leukemia) of new, "histocompatibility-like" antigens, such as have been reported by many investigators (*39*, *41–48*). It has been suggested, as an explanation for the extreme resistance of C57BL mice to the polyoma virus, that the virus sucessfully infects the cells of the new host, where it induces the formation of a new ("foreign") cellular antigen, resulting in rejection of those cells by the host (*30*, *42*, *43*). Presumably this new antigen would be coded for by the viral genome. Possibly such a code might derive from contamination with cellular genomic material involving a histocompatibility locus from a previous host by a transductive process. It might be of interest to test the tumorigenicity of polyoma virus in C57BL/10 and its congenic lines, and the transplantability in those strains of the resultant tumors, for evidence of isoantigenic specificity.

In summary, the data presented here indicate that the *H-2* region of the IXth linkage group in the mouse significantly influences host response to leukemogenesis by the B/T-L virus. There is some presumption that the influence of the *H-2* region is due to the *H-2* locus itself. If the effect of this region is due in whole or in part to other loci, they must necessarily be linked with *H-2*. While the *H-2* locus (or some other locus close to it) plays a demonstrable role in viral leukemogenesis in this system, it is clear that a number of other loci must also be important. Whether the non-*H-2* loci tested exert a significant effect remains uncertain. In any case, the over-all picture of susceptibility and resistance to viral leukemogenesis, as suggested by these data, is typical of those situations where multiple factors or loci determine the expression of a trait and where particular combinations of genes can produce well-defined phenotypic thresholds of expression. This structure would explain the apparent inconsistencies in the data and the partial effects of the "weaker" histocompatibility loci. In closing, it should be made clear that, although in this research and discussion the emphasis has been placed on the response and genetic constitution of the host, it is recognized that both host *and* viral genotype certainly contribute to the outcome of infection.

SUMMARY

The leukemogenicity of the BALB/Tennant-leukemia (B/T-L) virus for the C57BL/10 ScSn strain and 6 of its congenic lines was compared. Three of these lines, B10.A, B10.D2, and B10.BR, differ from the C57BL strain ($H\text{-}2^b$) by substitution at the *H-2* locus for the *b* allele, of the *a*, *d*, and *k* alleles, respectively. The effect of these substitutions in every case was to increase significantly susceptibility to leukemogenesis by the virus. At a 10^{-1} dilution of virus, the percentages of increase over C57BL/10 strain were 26, 32, and 22, respectively; at a 10^{-2} dilution, the relative increases were 29, 23, and 16. The most pronounced effect on susceptibility was conferred by $H\text{-}2^d$, the allele carried by the primary BALB/c strain. The presence of the *b* allele appeared to confer increased resistance. Three congenic strains [B10.C (41N), B10.C (47N), and B10.C (45N)] representing allelic substitutions at 3 loci derived from BALB/c (*H-1*, *H-7*, and *H-9*) were also challenged with the virus. At a 10^{-1} dilution of virus, 2 of these lines showed slight increases in susceptibility (6 and 11%), with an average latent period of 1 month less than for the C57BL/10 reference strain; at 10^{-2}, response was in the direction of increased resistance. The data for all strains, when challenged with the virus at a 10^{-2} dilution, indicate a considerable degree of resistance unaccounted for by the histocompatibility factors. In summary, the *H-2* region of the IXth linkage group of the mouse significantly influences

host response to the leukemogenic virus, but it is clear that multiple factors contribute to the total response of the host.

REFERENCES

(*1*) Gowan, J. W.: Experimental analysis of genetic determinants in resistance to infectious disease. Ann NY Acad Sci 91: 689–709, 1961.

(*2*) Hutt, F. B.: Genetic resistance to disease. *In* Animal Genetics. New York, Ronald Press, chapt 22, 1964, pp 500–526.

(*3*) Webster, L. T.: Inheritance of resistance of mice to enteric bacterial and neurotropic virus infections. J Exp Med 65: 261–286, 1937.

(*4*) Knudson, A. G.: Infection and immunity. *In* Genetics and Disease. New York, McGraw-Hill Book Co., chapt 6, 1965, pp 195–234.

(*5*) Law, L. W.: Genetic studies in experimental cancer. Advances Cancer Res 2: 281–352, 1954.

(*6*) Heston, W. E.: Genetics of neoplasia. *In* Methodology in Mammalian Genetics (Burdette, W. J., ed.). San Francisco, Holden-Day, 1963, pp 247–268.

(*7*) Waters, N. F., and Burmester, B. R.: Mode of inheritance of resistance to Rous sarcoma virus in chickens. J Nat Cancer Inst 27: 655–661, 1961.

(*8*) Crittenden, L. B., Okazaki, W., and Reamer, R. H.: Genetic control of responses to Rous sarcoma and strain RPL12 viruses in the cells, embryos, and chickens of two inbred lines. Nat Cancer Inst Monogr 17: 161–177, 1964.

(*9*) Dhaliwal, S. S.: Resistance of the chorio-allantoic membrane of chick embryos to Rous sarcoma and MH2 reticuloendothelioma viruses. J Nat Cancer Inst 30: 323–336, 1963.

(*10*) Waters, N. F., Burmester, B. R., and Walter, W. G.: Genetics of experimentally induced erythroblastosis in chickens. J Nat Cancer Inst 20: 1245–1256, 1958.

(*11*) Chang, S., and Hildemann, W. H.: Inheritance of susceptibility to polyoma virus in mice. J Nat Cancer Inst 33: 303–313, 1964.

(*12*) Little, C. C.: The genetics of spontaneous tumor formation. *In* Biology of the Laboratory Mouse (Staff, The Jackson Laboratory). New York, Dover Publications, Inc., chapt 6, 1941, pp 248–278.

(*13*) Lilly, F., Boyse, E. A., and Old, L. J.: Genetic basis of susceptibility to viral leukemogenesis. Lancet 2: 1207–1217, 1964.

(*14*) Tennant, J. R.: Susceptibility and resistance to viral leukemogenesis in the mouse. II. Response to the virus relative to histocompatibility factors carried by the prospective host. J Nat Cancer Inst 34: 633–641, 1965.

(*15*) MacDowell, E. C., Potter, J. S., and Taylor, M. J.: Mouse leukemia. XII. The role of genes in spontaneous cases. Cancer Res 5: 65–83, 1945.

(*16*) Cole, R. K., and Furth, J.: Experimental studies on the genetics of spontaneous leukemia in mice. Cancer Res 1: 957–965, 1941.

(*17*) Law, L. W.: The flexed-tail-anemia gene (*f*) and induced leukemia in mice. J Nat Cancer Inst 12: 1119–1126, 1952.

(*18*) Tennant, J. R.: Derivation of a murine lymphoid leukemia virus. J Nat Cancer Inst 28: 1291–1303, 1962.

(*19*) ———: Susceptibility and resistance to viral leukemogenesis in the mouse. I. Biologic definition of the virus. J Nat Cancer Inst 34: 625–632, 1965.

(*20*) Bronson, F. H., and Marsden, H. M.: Male-induced synchrony of estrus in deermice. Gen Comp Endocr 4: 634–637, 1964.

(*21*) Snell, G. D.,and Bunker, H. P.: Histocompatibility genes of mice. V. Five histocompatibility loci identified by congenic resistant lines on a C57BL/10 background. Transplantation 3: 235–252, 1965.

(*22*) Gross, L.: Biological and pathogenic properties of a mouse leukemia virus. Acta Haemat (Basel) 23: 259–275, 1960.

(*23*) Woolley, G. W., and Small, M. C.: Strain specificities of leukemia agent. Ann NY Acad Sci 68: 533–539, 1957.

(*24*) Kaplan, H. S.: Experimental alteration of host response to an occult tumor virus. Induction of thymic lymphosarcomas in X-irradiated mice. Nat Cancer Inst Monogr 4: 141–150, 1960.

(*25*) Moloney, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J Nat Cancer Inst 24: 933–951, 1960.

(*26*) Woolley, G. W., Eisenberg, R., and Mirner, R.: Further studies of susceptibility to Friend virus. Proc Amer Assoc Cancer Res 3: paper 297, 1962.

(*27*) Fieldsteel, A. H., Dawson, P. J., and Bostick, W. L.: Quantitative aspects of Friend leukemia virus in various murine hosts. Proc Soc Exp Biol Med 108: 826–829, 1961.

(*28*) Rauscher, F.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*29*) Möller, G., and Möller, E.: Phenotypic expression of mouse isoantigens. J Cell Comp Physiol 60 (Suppl): 107–128, 1962.

(*30*) Klein, G.: Genetic aspects of the relationship between viruses and tumor. *In* Proc 8th Int Congr Microbiol, 1963, pp 480–500.

(*31*) Kandutsch, A. A.: Intracellular distribution and extraction of tumor homograft-enhancing antigens. Cancer Res 20: 264–268, 1960.

(*32*) Kandutsch, A. A., and Stimpfling, J. H.: Partial purification of tissue isoantigens from a mouse sarcoma. Transplantation 1: 201–216, 1963.

(*33*) Snell, G. D.: Method for study of histocompatibility genes and isoantigens. Introduction. Meth Med Res 10: 1–7, 1964.

(*34*) Gye, W. E., and Purdy, W. J.: The infective agent in tumour filtrates: A further investigation by means of antisera to normal tissues. Brit J Exp Path 14: 250–259, 1933.

(*35*) Morgan, C., Howe, C., and Rose, H. M.: Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. J Exp Med 113: 219–234, 1961.

(*36*) Rubin, H.: Influence of tumor virus infection on the antigenicity and behavior of cells. Cancer Res 21: 1244–1253, 1961.

(*37*) Bernhard, W.: Some problems of fine structure in tumor cells. Progr Exp Tumor Res 3: 1–34, 1963.

(*38*) Harris, R. J. C.: Biological and structural properties of Rous sarcoma viruses. *In* 17th Ann Sympos on Fundamental Cancer Res. Baltimore, Williams & Wilkins Co., 1963, pp 331–343.

(*39*) Vogt, P. K.: The cell surface in tumor virus infection. Cancer Res 23: 1519–1527, 1963.

(*40*) Pettenkofer, H. J., Stöss, B., Helmbold, W., and Vogel, F.: In response to note of Springer, G. F., and Weiner, A. S., entitled: Alleged causes of the present-day world distribution of the human ABO blood groups. Nature (London) 193: 444–446, 1962.

(*41*) Habel, K.: Resistance of polyoma-virus-immune animals to transplanted polyoma tumors. Proc Soc Exp Biol Med 106: 722–725, 1961.

(*42*) Sjögren, H. O.: Studies on specific transplantation resistance to polyoma-virus-induced-tumors. II. Mechanism of resistance induced by polyoma virus infection. J Nat Cancer Inst 32: 375–393, 1964.

(*43*) Law, L. W.: Neoplasms in thymectomized mice following room infection with polyoma virus. Nature (London) 205: 672–673, 1965.

(44) Sachs, L.: Transplantability of an X-ray-induced and a virus-induced leukemia in isologous mice inoculated with a leukemia virus. J Nat Cancer Inst 29: 759–764, 1962.

(45) Klein, G., Sjögren, H. O., and Klein, E.: Demonstration of host resistance against isotransplantation of lymphomas induced by the Gross agent. Cancer Res 22: 955–961, 1962.

(46) Old, L. J., and Boyse, E. A.: Immunology of experimental tumors. Ann Rev Med 15: 167–186, 1964.

(47) Breyere, E. J., and Williams, L. B.: Antigens associated with a tumor virus. Rejection of isogenic skin grafts from leukemic mice. Science 146: 1055–1056, 1964.

(48) Jonsson, N., and Sjögren, H. O.: Further studies on specific transplantation antigens in Rous sarcoma of mice. J Exp Med 122: 403–422, 1965.

DISCUSSION

Dr. Klein: May I ask Dr. Tennant if she or anybody else has tested the possible effect of anti-H-2 antisera on viruses isolated from animal hosts of the corresponding *H-2* genotype. Is there any neutralizing effect?

Dr. Tennant: No, but these sera are now available in higher titer than they were at the time I was doing these studies and this is projected. Actually I'm receiving the serum this coming week.

Dr. Rowe: I have a technical question for Dr. Tennant. I would like to ask her what measures were taken to be sure the dosages of virus given to the various congenic lines were exactly comparable. Also, I could not tell whether you were comparing data obtained at markedly different times.

Dr. Tennant: Yes, definitely comparable. The passages were the same and this is in the text of the manuscript. Whenever a virus pool was changed, or whenever a shipment of mice was inoculated, both BALB/c and C57BL/10 babies were inoculated at the same time, with the same virus, at the same dilution.

Dr. Rowe: When you are trying to attribute significance to the difference between 85% and 62% responses, it takes both very large numbers of animals and careful control of possible variables.

Dr. Tennant: Yes. As I say, any time a group of animals was to be inoculated, both of those control groups were inoculated with exactly the same material at the same time and same dilution.

Dr. Breyere: I'd like to ask Dr. Tennant two short questions. First of all, have you ever worked with the relatively resistant NH, or New Haven, strain of mice? Secondly, do you by chance know their *H-2* phenotype?

Dr. Tennant: No, I have not, and no, I do not.

Inhibition of Virus-Induced Leukemia in Mice by a Nontumor Virus[1]

E. Frederick Wheelock, *M.D.*, *Ph.D.*, *Associate Professor of Preventive Medicine*, *Western Reserve University*, *Cleveland*, *Ohio*

THE virus etiology of certain murine leukemias and possible virus etiology of human leukemia make it reasonable to think of treatment of these diseases in terms of virus inhibition. One means of inhibiting virus replication in cells is by the introduction of a second virus which can either compete with the first virus for replicating sites or induce synthesis of interferon, a virus inhibitor.

In recent years viral interference has been demonstrated in a variety of nontumor virus-cell culture and animal systems. In contrast to nontumor viruses, there has been great difficulty in obtaining growth of murine leukemia viruses in cell culture systems, and *in vitro* studies of viral interference with this class of viruses have not been conducted. Such studies, however, are possible in *in vivo* systems.

The purpose of the present investigation was to study the effects of inoculation of nontumor viruses on virus-induced leukemia in mice and to determine the mechanism of any beneficial changes produced in the leukemic disease by these viruses. In the experiments reported herein a reciprocal interference was found in DBA/2 mice between Sendai virus (one of the parainfluenza group of viruses which produces benign infections in mice) and Friend leukemia virus.

Friend leukemia virus was obtained from Charlotte Friend in the form of infected DBA/2 mice. Vesicular stomatitis virus was grown in "L" cell monolayer cultures. Sendai virus was grown in the allantoic sac of 10-day-old embryonated chicken eggs. The "L" strain of mouse fibroblasts derived from normal mouse skin was employed in the assay for interferon.

The specimens to be tested for interferon were diluted in growth medium, and 1 ml of each dilution was added to 1-day-old cultures of "L" cells grown in incomplete monolayers in screw-cap tubes. After 20-hour incubation at 37° C, the cultures were washed once with 4 ml of phosphate-buffered saline. One ml of warm Eagle's medium was added to each tube, and then 1 ml of cold growth medium containing 5000 tissue culture infective doses (TCID50) of vesicular stomatitis virus was inoculated. This amount of virus produces gross cytopathic effects in cultures in 24 to 30 hours. Cultures were considered to be protected when there was less than 10% cytopathic effect at a time when control cultures exhibited more than 75% cytopathic effect.

---

[1] Summary of paper presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

Six- to 8-week-old DBA/2 female mice (purchased from The Jackson Laboratory) were anesthetized with ether and exsanguinated, and the abdominal wall was shaved. The peritoneal cavity was opened and washed with 5 ml of phosphate-buffered saline. The recovered saline wash was then clarified by centrifugation at 2000 rpm for 5 minutes, and the supernatant was acidified at *p*H 2 for 24 hours, then neutralized and processed as described above for the interferon assay.

Sendai virus inoculated into DBA/2 mice as long as 6 weeks prior to Friend virus markedly inhibited the splenomegalic response to Friend virus. This inhibitory effect depends on the route of inoculation; both viruses must be administered intraperitoneally. Newcastle disease, vesicular stomatitis, and Sindbis viruses had *no* such Friend virus inhibitory properties.

Sendai virus inoculated into mice intraperitoneally induces the production in the peritoneal cavity of $\approx 10^3$ units of interferon within 24 hours. Within 1 day after Friend virus inoculation intraperitoneally or intravenously, mice undergo a marked reduction of interferon response to Sendai virus inoculation. By 12 days after Friend virus inoculation, mice can once again synthesize normal amounts of interferon in response to Sendai virus. Details of these experiments can be found in the *Proceedings of the National Academy of Sciences, USA* 55: 774, 1966. In more recent experiments it has been found that the inhibition of Friend virus splenomegaly by prior inoculation with Sendai virus is associated with a marked reduction in Friend virus titer per mg of spleen.

The reciprocal interference between Sendai and Friend viruses demonstrates that nontumor viruses have the capacity both to inhibit and be inhibited by leukemia viruses. It should be pointed out that the interference of Sendai against Friend virus need not involve the same mechanism as that of Friend against Sendai. However, primary peritoneal host cells common to both viruses could result in competition between the two viruses for cellular replicating sites and lead to both types of interference. This possibility is experimentally approachable and is under current investigation. Other explanations of the observed interference could be serological relationships between Sendai and Friend viruses and a direct oncolytic effect of Sendai virus on Friend virus-infected cells. That Sendai virus inoculated 6 weeks prior to Friend virus can still inhibit splenomegaly in a significant number of mice makes the role of interferon unlikely as the mediator of virus interference.

The temporary loss of the ability to produce interferon in response to Sendai virus in mice following Friend virus inoculation may be an important factor in the pathogenesis of the leukemic disease. Friend virus in the early stages of infection may either occupy critical cellular sites or disrupt biosynthetic mechanisms of interferon production in peritoneal cells. Whether the loss of interferon production is necessary for the establishment of Friend infection is problematic. However the transient nature of the loss may mean the infection once established can persist even in the presence of interferon. This hypothesis is supported by the observation that Sendai virus will inhibit the splenomegalic response to Friend virus only when given before but not after Friend virus. Interestingly, Sendai virus did not inhibit splenomegaly even when given after a return of the interferon-producing function, viz., by the 12th day after Friend virus inoculation. However, the spleens by this time are rapidly enlarging and thus perhaps refractory to inhibition.

If the interference between Sendai and Friend viruses demonstrated in the laboratory system described herein has a counterpart in nature, it may be in the form of an interference between certain nontumor viruses and leukemia viruses. Thus, in nature there may be constant inhibition of leukemia viruses by other viruses. Emergence of a leukemia virus into a disease-producing agent may be the result of either the removal of the inhibiting viruses or a breakdown in the interfering mechanism between viruses. The study in mice of a leukemia virus that is partially inhibited by the prior inoculation of a nontumor virus may assist in the discovery and understanding of such a balance between viruses in nature.

DISCUSSION

Dr. Latarjet: You speak of interferons measured on the basis of the reduction of splenomegaly on the 28th day. What is the final outcome of those animals that have undergone these reductions? Do they develop the Friend disease or do they not, and do they die and when?

Dr. Wheelock: We are engaged in such an experiment in which we have varied two things. We have varied the interval between Sendai virus and Friend virus to see how long a period can elapse between inoculation of one and the other with subsequent inhibition of the splenomegalic response to Friend virus. These experiments are in progress. We now find that at a 6-week point between Sendai virus and Friend virus we still get in some mice significant reduction in the splenomegalic response to Friend virus. Specifically in answer to your question, when we inoculate mice with Sendai virus and 7 days later give them Friend virus and then sacrifice mice at 3-week intervals thereafter to see the course of the disease in these mice, we find that at a 9-week point there is still reduction in the size of the spleen compared with Friend virus control mice. In addition, Sendai virus inoculated into mice prior to Friend virus markedly prolongs the mean survival time as compared with Friend virus controls. In addition, 10% of mice inoculated with both viruses were still alive and manifesting no evidence of leukemia 8 weeks after the last Friend virus control mouse had died.

Dr. Metcalf: This is a question related to Dr. Latarjet's comment. It seems to me the choice of the 3- or 4-week endpoint for splenomegaly is a rather unfortunate one because it prevents discriminating between the various possibilities. Does the virus get inside the susceptible cell? Does the susceptible cell proliferate? Does it become neoplastic?

The initial response to the Friend virus, the very first visible response, is the proliferation of reticulum cells in the spleen in little foci along the trabeculae of the spleen. This is seen best perhaps at day 3 or day 5 (Metcalf D., Furth, J., and Buffett, R. F., Cancer Res. 19: 52, 1959), and I suggest that this might be a more suitable endpoint, a little more laborious, if you like, since it involves histological examination but suitable in providing information as to whether the virus actually gets in and stimulates this initial cell proliferation.

Dr. Rowe: I want to ask Dr. Wheelock if he knows whether his mouse strain is free of Sendai. This virus is so prevalent in positive colonies that it would be a serious complicating factor if it were present.

Dr. Wheelock: Yes, I know that colonies of mice have been found to be chronically infected with Sendai virus. I have not tested my mice to determine if they are immune to Sendai virus. This is a constant worry, but at the moment since Sendai virus appears to be producing an inhibitory effect on Friend virus leukemia we just proceed on the assumption that these mice are not immune to this virus.

Dr. Rowe: Some years ago we did some interference experiments rather similar to this, but did not use Sendai. We were interested in what might happen if our passage Friend virus became contaminated with another virus, so we studied the effect of mixing Friend with other mouse viruses. K virus, mouse adenovirus, mouse hepatitis (MHY–1), and thymic agent had absolutely no effect on spleen response, but lymphocytic choriomeningitis virus (LCM) essentially prevented splenomegaly. We also compared the susceptibility to Friend virus of mice congenitally infected with LCM and mice of the same genetic line (but bred separately for 20 years). The carrier mice were definitely susceptible to Friend, but about a log less sensitive than the noncarrier strain, which I attribute to genetic differences rather than LCM carrier status.

We found the same pattern with LCM in interference with many other viruses. LCM as an acute infection of an infant or adult mouse is an extremely potent interferer; it interferes with many lethal viruses inoculated about a week later. But the carrier mice born with high titer to virus are absolutely normal in their response to the same lethal viruses. Thus interference seems to be an acute response that is not continually produced during a chronic infection, at least with this one virus.

Dr. Bases: I want to suggest, Dr. Wheelock, that the "intrinsic interference" which Dr. P. I. Marcus and Dr. David Carver found (Science 149: 983–986, 1965) may perhaps be at the basis of the interference you have described between Sendia and Friend viruses. Previous infection of a variety of cells by rubella virus or Sindbis virus gave complete protection to challenge with Newcastle disease virus (NDV) *in vitro*. This does not seem to be interferon mediated and may be an entirely different mechanism of interference.

Incidentally, I have recently tested the chronically infected BALB line, JLS V–5, and its parent cell, line JLS V–6, for sensitivity to NDV *in vitro*. It has been rather rare to find cells resistant to NDV in cell culture. Mouse, rat, hamster, and even frog cells were sensitive. Interestingly enough, the Rauscher carrier line was uniformly resistant to challenge with NDV, whereas the original cell line JLS V–6 was entirely sensitive as measured by cytopathogenic effect and also by the hemadsorption test. Interference between certain tumor viruses and myxoviruses may be a real possibility.

MORPHOLOGY AND DEVELOPMENT OF MURINE LEUKEMIA VIRUSES

Chairman: LEON DMOCHOWSKI

Origin of the Viremia in Murine Leukemia [1,2]

Etienne de Harven *and* Charlotte Friend,[3]
Sloan-Kettering Institute for Cancer Research,
New York, New York 10021

Little is known concerning the earlier phases of the *in vivo* multiplication of murine leukemia viruses. The identity of the first target cells is still mysterious. The duration of the intracellular cycle of the virus has not as yet been determined. Study of these questions is complicated because most strains of mice are chronically infected with morphologically similar latent viruses, particularly visible within the epithelial cells of the thymuses (*1*). Another difficulty originates from the fact that direct infection of tissue cultures with Friend leukemia viruses has met with limited success (*2*). Cell lines producing murine leukemia viruses have occasionally been established by adapting to *in vitro* conditions cells from previously infected mice or chronically infected transplantable murine tumors. In such material, the "time zero" of the infectious process can scarcely be determined.

Four days after adult DBA/2 mice have been inoculated intraperitoneally with a cell-free preparation of the Friend virus, large numbers of virions easily identified by electron microscopy appear in the plasma of the infected animals. At that time, the infectivity of the plasma filtrates can be readily demonstrated by bioassay in susceptible mice. These findings agree with those found in the study of other murine leukemia viruses.

Our present studies, based on a strict correlation between fine structure and bioassays, do not answer completely the question of the origin of the viremia. However, in our efforts to clarify this problem, several unexpected observations were made that are the subject of the present report.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported by Public Health Service grants NCI-183 and CA-02062 from the National Cancer Institute, General Research Support Grant NIH 1-SO1-FR05534 from the National Institutes of Health, and NYC HRC contracts I-325 and U-1096II C2.

[3] The skillful technical assistance of Mrs. C. Jamieson, Miss N. Lampen, and Mr. G. Holland is gratefully acknowledged as well as the secretarial assistance of Miss P. Baldwin in the preparation of the manuscript.

MATERIALS AND METHODS

Virus.—Filtrates of 20% homogenates of leukemic spleens were prepared as previously described (*3*). Each mouse was inoculated intraperitoneally with 0.2 ml of filtrate.

Mice.—DBA/2 mice from The Jackson Laboratory, Bar Harbor, Maine, 4 to 8 weeks old, of either sex were used throughout this study.

Bleeding.—The blood was collected from the axilla, immediately diluted with Ringer's solution containing 4 units of heparin per ml, and chilled in ice.

Purification of the virus (*4–7*).—The pooled, diluted blood from approximately 20 mice was first centrifuged at 1500 rpm (International) for 20 minutes. The plasma was collected and subjected to two successive filtrations, first through a 0.65 μ and second through a 0.22 μ Millipore membrane. The last filtrate was spun in 6 ml tubes at 30,000 × *g* (Spinco model L, rotor 40) for 2 hours. The same technique was applied to tissue culture fluids.

Tissue culture.—A cell line (*8*), designated C–1A, propagating in suspension cultures in Eagle's basal medium with Earle's balanced salt solution and supplemented with 10% fetal calf serum, was used.

Peritoneal macrophages.—The peritoneal cavity of the mice was washed with 5 to 10 ml of heparinized Ringer's solution (4 units/ml) for collection of peritoneal macrophages.

Preparation of tissues for electron microscopy.—Samples of spleen, liver, thymus, and bone marrow were fixed in 2% buffered glutaraldehyde, postfixed in 1% buffered osmium tetroxide, and occasionally treated with 0.5% uranyl acetate at *p*H 5 (*9*) before ethanol dehydration and embedding in Epon 812. Thin sections were stained with uranyl acetate followed with lead hydroxide, coated with a carbon film, and examined in a Siemens Elmiskop IA electron microscope occasionally equipped with an anticontamination device cooled with liquid nitrogen. Pellets of purified viruses or of tissue culture cells were prepared essentially the same way, except for 1% instead of 2% glutaraldehyde, and for shorter fixation times.

OBSERVATIONS

Pellets Prepared From the Plasma of Virus-Inoculated DBA/2 Mice

Three groups of approximately 100 mice each were inoculated with the virus. At 1, 2, 3, 4, 7, 14, and 21 days after treatment, the blood of 20 to 25 of these animals was collected and pellets of the filtered plasma were prepared for electron microscope study and bioassays.

No leukemia virus was observed in the pellets prepared 1 and 2 days after inoculation. However, extremely large numbers of *a smaller particle* were seen at that time (figs. 1, 2, and 3). These particles measure approximately half the size of leukemia virus particles. They seem to form two different categories. Most of them have a rather indistinct oblong shape as seen in figure 3. Such particles are approximately 70 mμ long and 35 mμ wide. Their peripheral envelopes are poorly outlined. They contain a denser core with an electron-lucent center. Another pellet, also prepared 2 days after inoculation, showed more spherical and better preserved particles (figs. 1 and 2). These particles are spherical, their diameter averages 57 mμ, and their envelope shows a definite unit-membrane structure (fig. 2). A small and electron-dense doughnut-shaped core, 25 to 28 mμ in diameter, is regularly observed within these particles. The fact that in several cases (figs. 1 and 2) all the small particles are spherical seems to rule out the possibility that they simply represent cross-sections of the elongated forms. More complete fixation procedures were applied in these cases and this perhaps accounts for the differences seen between the oblong and the spherical forms.

The structure of these small particles is a miniature version of classical type A1 leukemia viruses. Their size, however, makes any identification of these small particles with a leukemia agent most doubtful.

Since it is known that mice developing Friend leukemia are frequently superinfected with the lactic dehydrogenase (LDH) agent (*10*), and since, on the other hand, the structure of the observed small particles is almost identical to that of LDH viruses recently described by de-Thé (*11*) and Notkins (*12*), it is reasonable to assume that at days 1 and 2 after inoculation we observed a peak of LDH viremia, nonspecifically related to the leukemia, but indicating the contamination of our leukemia virus strain.

At 3 days after inoculation, the plasma pellets still showed a few LDH viruses, while the first typical 100 mμ leukemia viruses appeared. No transitional form between the LDH agent and the leukemia virus was observed in this material. On day 4, large numbers of leukemia virus were seen, occasionally mixed with vesicular cell debris and platelet granules. From the 4th day on, findings remained comparable until the end of the experiment.

As discussed earlier (*5*), purified virions in Friend leukemia consist mostly of type C particles, with only 2 to 4% of type A1 particles. Although slight variations in the A1/C ratio are frequently observed, apparently even at the time of their earliest appearance in the bloodstream (day 3 or 4), most of the particles are type C. On the fourth day, 2.3% of A1 particles was counted. In figure 4, illustrating a pellet from the 7th day, 1.3% of A1 particles was observed.

The large number of particles seen on day 4 or 7 correlates with the level of biological activity reached at that time.

Tissues of Virus-Inoculated Mice

The cellular origin of the plasma virions is still obscure. Apparently many different cells, not only leukemic ones, can differentiate leukemia viruses along their plasma membranes. Since the involvement of megakaryocytes in leukemia virus multiplication was first demonstrated (*13*), these cells have received much attention as potential virus producers. Platelets also were investigated (*14*), because, when released from virus-infected megakaryocytes, they can act in the bloodstream as virus carriers.

Samples of bone marrow, spleen, thymus, and circulating leukocytes were studied in an attempt to localize the viral factories responsible for the onset of the viremia. Since 4 days after inoculation the viremia has almost reached its height, attention is being focused on earlier phases, soon after inoculation. From the 4th day on, budding viruses can be observed almost everywhere and particularly in the megakaryocytes, platelets, spleen, and thymuses of the virus-inoculated mice. But these phenomena seem to accompany the development of the viremia rather than to initiate it.

The only remarkable observation made during the early phases *preceding* the onset of the viremia concerns the thymus. One and 2 days after inoculation, several thymuses have shown an unusual number of A1 particles, accumulated within the cytoplasmic vacuoles of thymic epithelial cells (fig. 5). The significance of such observations is hard to evaluate, since even apparently healthy mice will show such particles associated with these cells (*1*). It is difficult to quantitate such findings and to determine whether the thymic particles reflect a sudden acceleration in the multiplication of particles present before infection, or whether they are directly related to the intraperitoneal inoculation of viruses 24 hours earlier. Moreover, it has been reported that thymectomized mice are susceptible to Friend leukemia (*15*). Nevertheless, it might be interesting to reopen the question and to find out if thymectomized mice inoculated with the virus will become viremic as rapidly as control, nonthymectomized animals.

In one case, budding of A1 particles was observed in a spleen 2 days after inoculation. Bone marrow megakaryocytes observed 1 or 2 days after inoculation did not produce virus, though these cells actively produced virus 4 days after inoculation and for the subsequent course of the disease.

Peritoneal Macrophages

Peritoneal macrophages were collected 1 and 2 hours after the intraperitoneal inoculation of the virus. Evidence was obtained showing that rare C type particles were phagocytized by these cells. Many viruses penetrate cells by a phagocytic process as a first step in a multiplication cycle (*16*), and it is tempting to assume that this is one more example of the same phenomenon. More data, however, will have to be collected before any conclusion can be reached. So far, it is unknown whether the phagocytized viruses participate in the viral leukemic transformation of our mice, or, on the contrary, if they correspond to an inactivated part of the inoculum.

"LDH-Free" Experiments

When it was realized that a peak of LDH viremia repeatedly preceded the leukemia viremia, experiments were designed to free our Friend virus inoculum of the LDH agent by passage in rats (*17*) or by use of tissue culture material.

Attempts were made to recover the leukemia virus 3, 5, and 7 days after its inoculation to young A × C rats. On day 3, the LDH agent was still present. On days 5 and 7 it had disappeared, according to the results of plasma infectivity tests.[4] Unfortunately, at these times, the amount of leukemia virus recovered was insufficient for a large scale study in mice.

Results have been more encouraging with our tissue culture material. Here again, several unexpected findings were made that will now be described.

C-1A Tissue Cultures

The C-1A suspension cultures obviously represent a complex population of cells. Most cells are of the reticulum stem cell type, strongly reminiscent of the tumor cells from which these cultures originated. In addition, typical erythroblasts are seen at various phases of their maturation. Detailed characterization of these erythropoietic cells is given elsewhere (*8*). These cells, illustrated in figure 7, have the high cytoplasmic density and the particular chromatin distribution of erythroblasts.

Figure 8 illustrates the typical viral budding activity at the surface of the stem cells. The particles in the extracellular spaces were almost exclusively of the A1 type. Viruses were also observed in the vacuolated cytoplasm of a few erythroblasts.

Most interesting was the study of the pellets prepared from the filtered culture media. As illustrated in figures 9 and 10, such pellets consist of a remarkably pure collection of A1 particles with only very rare particles of the C type. The structure of these A1 particles was identical with previously described "young" particles, *i.e.*, particles immediately after their release in extracellular spaces. This finding seems to have some special significance, since another cell line, the V5, chronically infected with the Rauscher virus (*18*),[5] apparently elaborates large numbers of viruses, most of them of the C type, accompanied by only rare A1 particles as seen in figure 6.

Although the pellets obtained from the filtered fluids of C-1A cultures appear to have a high degree of purity, as judged by electron microscope observation, it was necessary to centrifuge large volumes of fluid to recover almost invisible pellets. The biological activity of A1 particles remains a matter of discussion. They have frequently been considered as a "younger" form of the virus (*19*), but not necessarily immature. However, C particles have been hypothetically considered as already inactivated virions (*5*), though Dalton considers them to be the infective particles (*14*). Because it was impossible to separate the A1 from the C particles, these questions remained open. Since preparations extremely rich in A1 particles now can be obtained from the C-1A cultures, some progress in this direction may be expected.

---

[4] We are grateful to Mr. V. Riley who kindly tested the LDH elevating activity of our preparations.

[5] We are grateful to Dr. G. Barski for kindly providing us with this cell line.

So far, we have been unsuccessful in our efforts to evaluate adequately the biological activity of these preparations by our routine titering *in adult Swiss mice.* However, when newborn mice or highly susceptible adult DBA/2 mice are inoculated with the same material, leukemia develops in a high percentage of the animals. Moreover, pellets from filtered culture fluid are as active as the cultured cells in protecting mice against the challenge with our original strain of leukemia virus (Friend: to be published). Apparently A1 particles have at least some of the antigenic determinants of the mature virus. The difficulty in demonstrating their infectivity is possibly related either to the small number of the particles recovered from the culture fluids or to the "incomplete" nature of A1 particles. That an essential step in the maturation of the virus (from "incomplete" to "infective") could occur outside the cells is difficult to conceive because of the strict dependence of virus synthesis on cellular enzymatic systems. Therefore, we are inclined to favor the first explanation and to incriminate the small number of A1 particles for the weak infectivity of these preparations.

DBA/2 mice treated with three consecutive injections of this material developed typical splenomegaly. Such spleens were negative for the LDH agent. Their inoculation to adult DBA/2 mice resulted in no visible LDH viremia 1, 2, 4, and 7 days after treatment.

Finally, when a plasma pellet was prepared from the blood of DBA/2 mice showing splenomegaly 3 to 4 weeks after repeated treatments with highly purified A1 particles, a typical viremia mostly of C particles was easily demonstrated.

SUMMARY AND CONCLUSIONS

Tissues of DBA/2 mice were examined under the electron microscope at various intervals (1–21 days) after inoculation with the Friend virus. Pellets of the filtered plasma were also examined for study of the development of the viremia. Four days after inoculation, when spleen enlargement was already apparent, large numbers of leukemia viruses, most of them type C, were observed free in the blood plasma. At that time, many cell types were participating in the process of virus multiplication, as shown by the large number of budding particles. Therefore, the localization of the first viral factories should be sought soon after infection. So far, the only suggestive observation has been the presence of many type A1 particles in the epithelial cells of the thymuses, as soon as 1 or 2 days

after inoculation. Rare leukemia viruses have been observed in the plasma 3 days after inoculation. Before that time, a remarkable and apparently short-lived peak of LDH agent viremia was observed, which indicated that our line of Friend virus is contaminated by this smaller agent.

The C-1A suspension cultures of cells originating from a solid leukemic tumor will be valuable material for further studies. These cultures represent a complex population of stem cells and erythroblasts. Both cell types produce typical A1 particles, and *almost pure preparations of A1 particles can be recovered from the filtered culture fluid.* These preparations are highly antigenic and immunize mice efficiently against challenge with the leukemia virus. Their apparently weak biological activity may be attributable to the small number of particles recovered from culture fluids. These preparations of A1 particles are free of LDH agent and will be further investigated to elucidate the relationship of A1 to C type particles as well as the complex effects of the virus on stem cells and on the erythropoietic system.

REFERENCES

(*1*) de Harven, E.: Virus particles in the thymus of conventional and germfree mice. J Exp Med 120: 857–868, 1964.

(*2*) Moore, A. E.: Growth and persistence of Friend leukemia virus in tissue culture. J Nat Cancer Inst 30: 885–895, 1963.

(*3*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307–318, 1957.

(*4*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*5*) de Harven, E.: Viremia in Friend murine leukemia: The electron microscope approach to the problem. Path Biol 13: 125–134, 1965.

(*6*) de Harven, E., and Friend, C.: Structure of virus particles partially purified from the blood of leukemic mice. Virology 23: 119–124, 1964.

(*7*) Friend, C., and de Harven, E.: A new method for purifying a murine leukemia virus. Fed Proc 24: 266, 1965.

(*8*) Friend, C., Patuleia, M. C., and de Harven, E.: Erythrocytic maturation *in vitro* of murine (Friend) virus-induced leukemic cells. Nat Cancer Inst Monogr 22: 505–522, 1966.

(*9*) Farquhar, M. G., and Palade, G. E.: Cell junctions in amphibian skin. J Cell Biol 26: 263–291, 1965.

(*10*) Riley, V., Lilly, F., Huerto, E., and Bardell, D.: Transmissible agent associated with 26 types of experimental mouse neoplasms. Science 132: 545–547, 1960.

(*11*) de-Thé, G.: Ultrastructure of the lactic dehydrogenase virus (LDV) and cell-virus relationships. Virology 26: 512–516, 1965.

(*12*) Notkins, A. L.: Lactic dehydrogenase virus. Bact Rev 29: 143–160, 1965.

(*13*) de Harven, E., and Friend, C.: Electron microscope study of a cell-free induced leukemia of the mouse: A preliminary report. J Biophys Biochem Cytol 4: 151–156, 1958.

(*14*) Dalton, A. J., Law, L. W., Moloney, J. B., and Manaker, R. A.: An electron microscopic study of a series of murine lymphoid neoplasms. J Nat Cancer Inst 27: 747–791, 1961.

(*15*) Metcalf, D., Furth, J., and Buffett, R. F.: Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res 19: 52–58, 1959.

(*16*) Dales, S.: Penetration of animal viruses into cells. Progr Med Virol 7: 1–43, 1965.

(*17*) Mahy, W. J., Rowson, K. E. K., Salaman, M. H., and Parr, C. W.: Plasma enzyme levels in virus-infected mice. Virology 23: 528–541, 1964.

(*18*) Wright, B. S., and Lasfargues, J. C.: Long-term propagation of the Rauscher murine leukemia virus in tissue culture. J Nat Cancer Inst 35: 319–327, 1965.

(*19*) de Harven, E.: Ultrastructural studies on three different types of mouse leukemia: A review. *In* Tumors Induced by Viruses: Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York, Academic Press Inc., 1962, pp 183–206.

PLATES

PLATE 1

FIGURE 1.—Plasma pellet, 2 days after inoculation. Among a variety of vesicular cell debris, small dense particles are seen, believed to represent the LDH agent. × 32,500

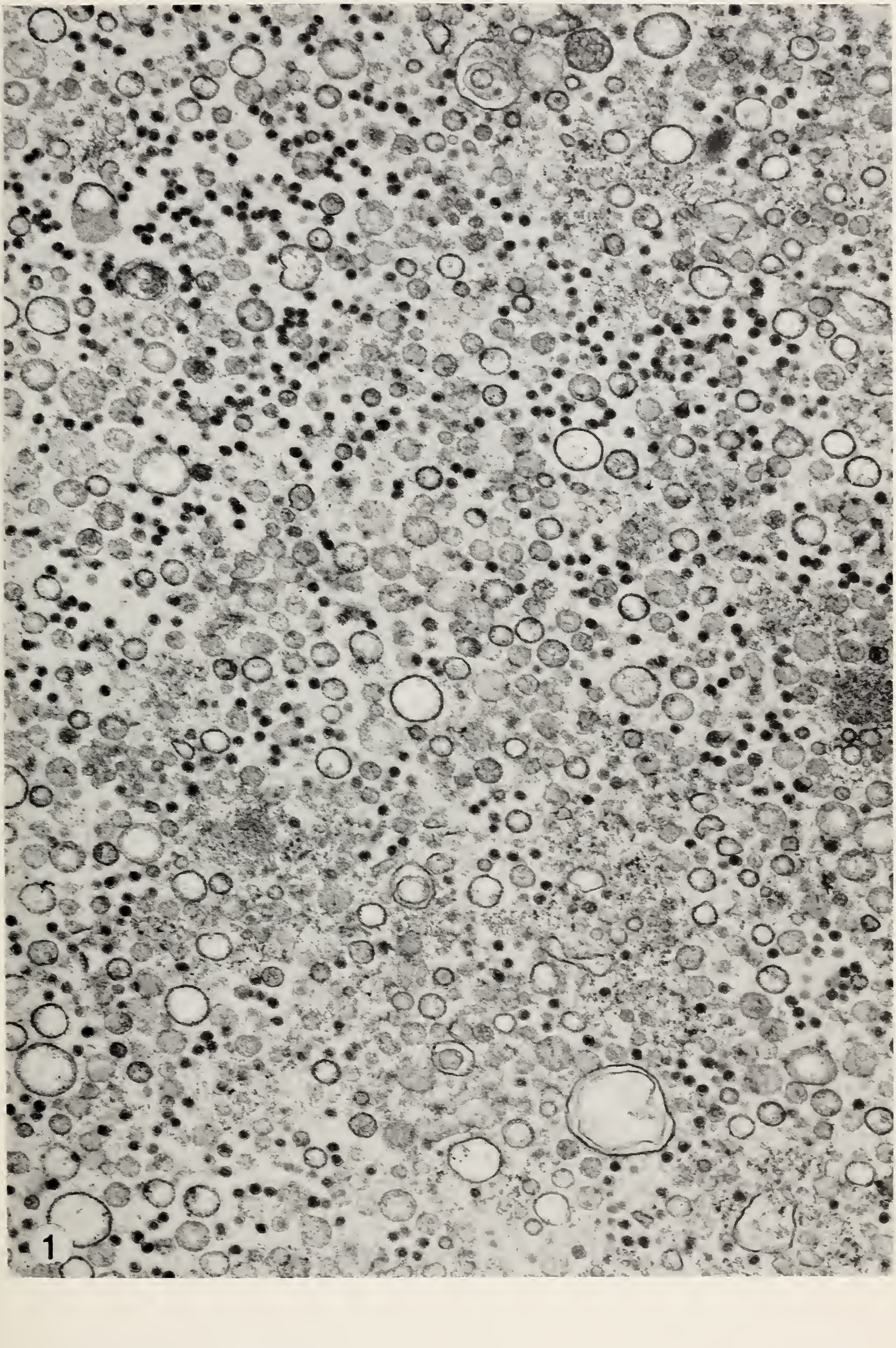
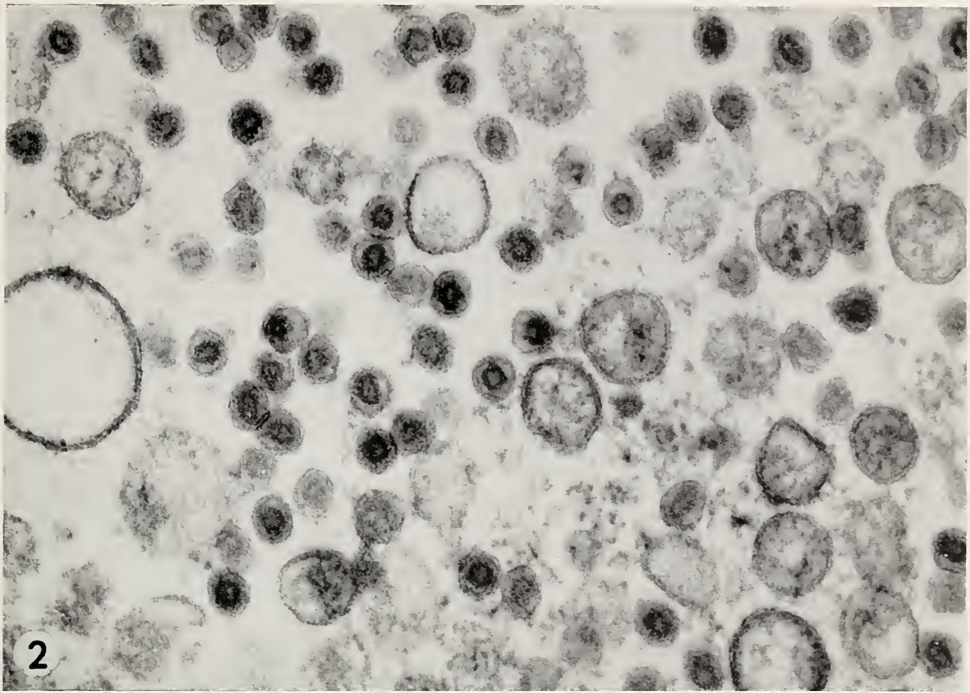

1

PLATE 2

FIGURE 2.—Same material as in figure 1 at higher magnification, showing unit membrane structure of external envelope of viruses and dense doughnut-shaped core. × 105,000

FIGURE 3.—Plasma pellet, 1 day after inoculation, showing oblong small particles of poorly delineated contour and believed also to represent the LDH agent in an earlier developmental phase or less adequately preserved during fixation procedure. × 90,000


2

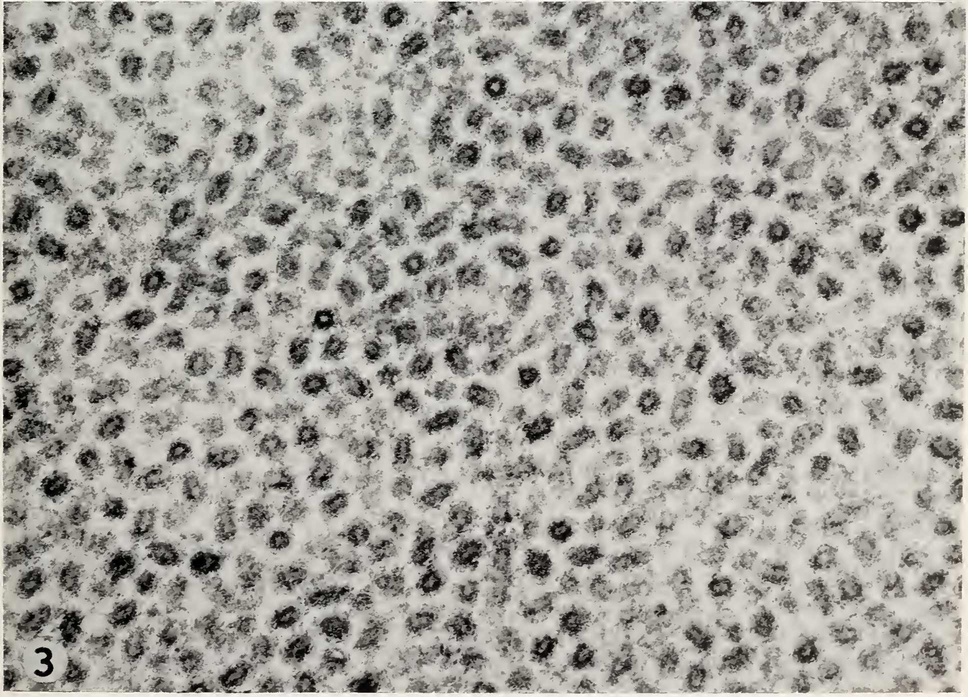
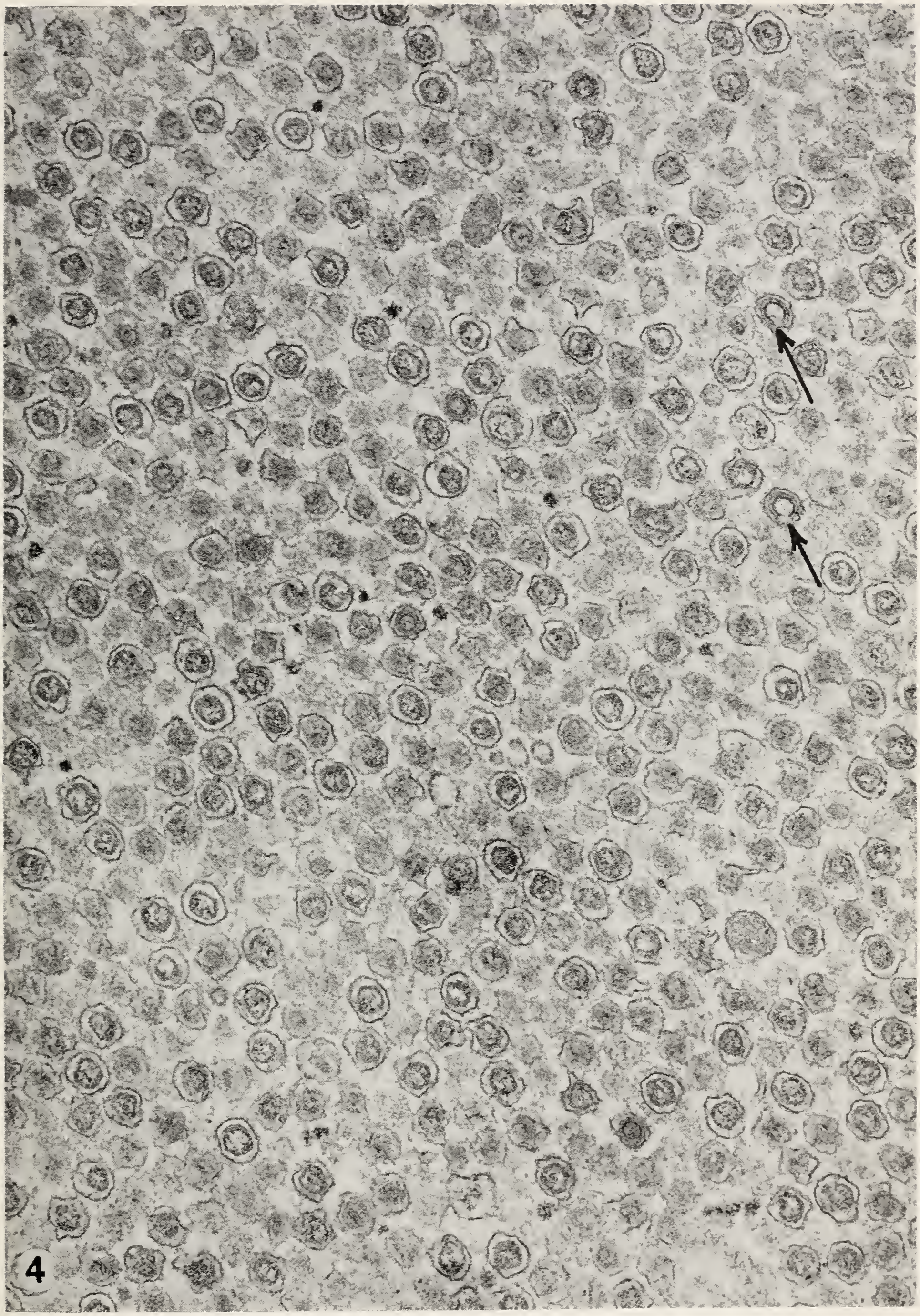

3

PLATE 3

FIGURE 4.—Plasma pellet, 7 days after inoculation with virus. An almost pure collection of Friend leukemia viruses is seen, consisting of mostly type C particles. *Arrows* indicate rare type A1 viruses. × 52,000

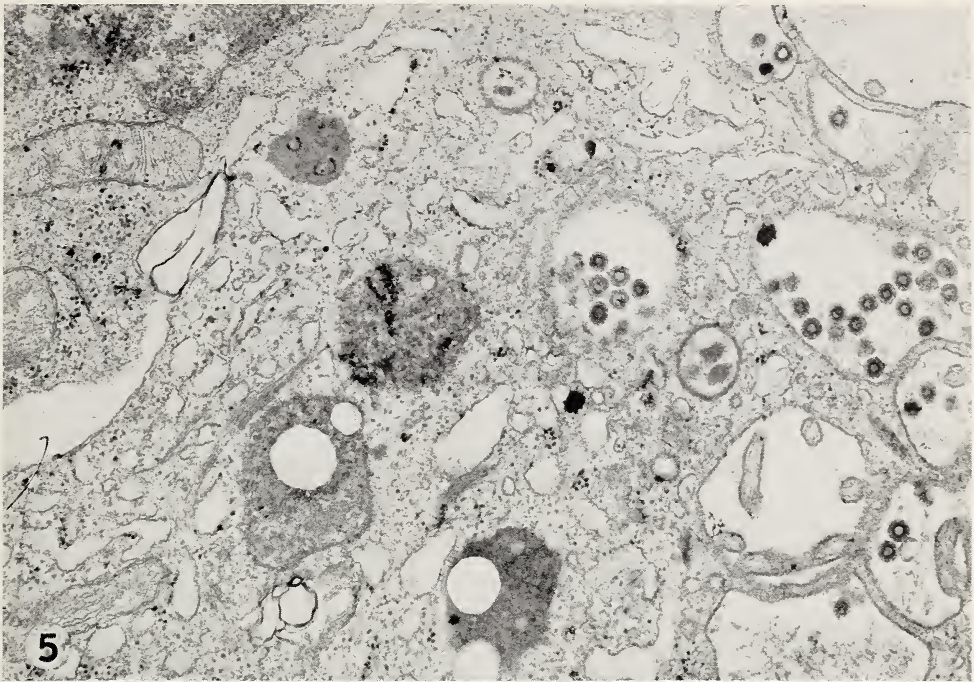


4

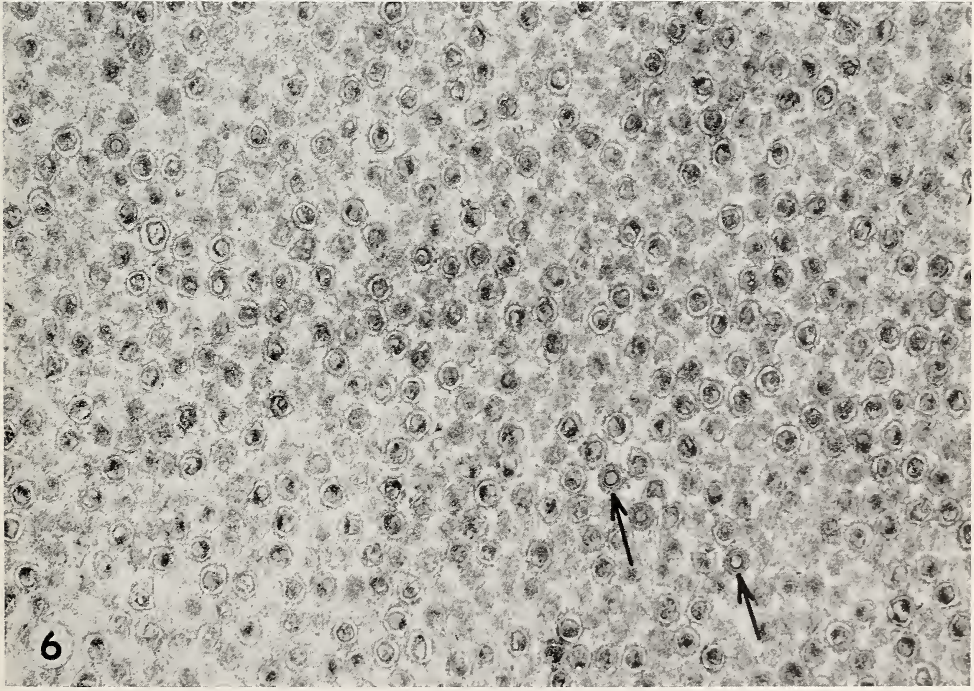
Plate 4

Figure 5.—Thymic epithelial cell of a DBA/2 mouse *1 day* after inoculation with Friend virus. *Note* accumulation of 100 mμ A1 particles in cytoplasmic vacuoles. × 30,000

Figure 6.—Pellet prepared from filtered medium of V5 tissue cultures. Many C particles (Rauscher virus) are seen. *Arrows* point to rare A1 particles. × 40,000



5

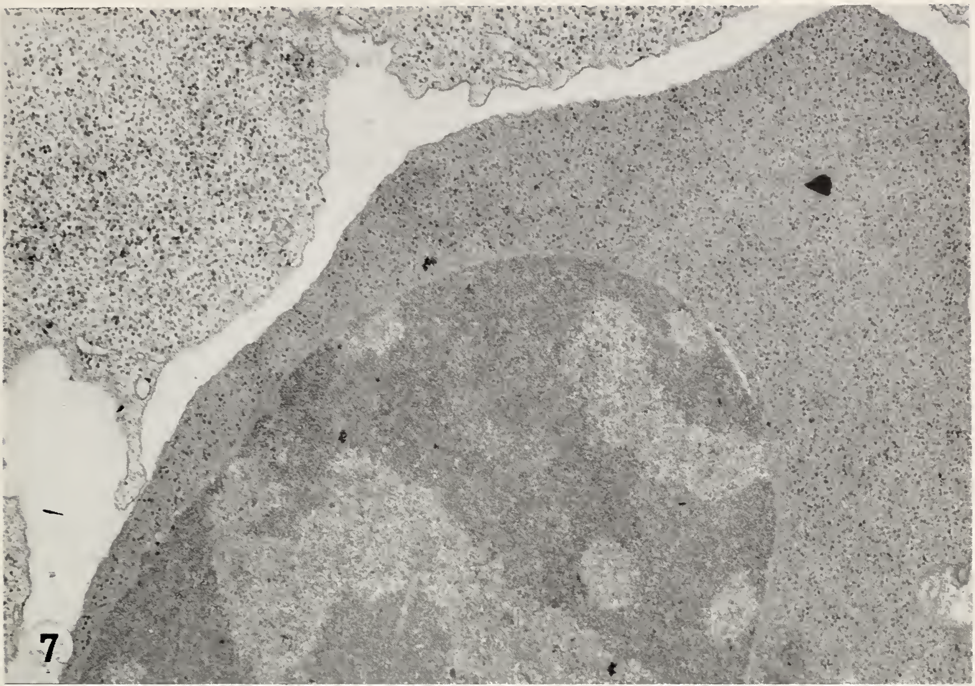


6

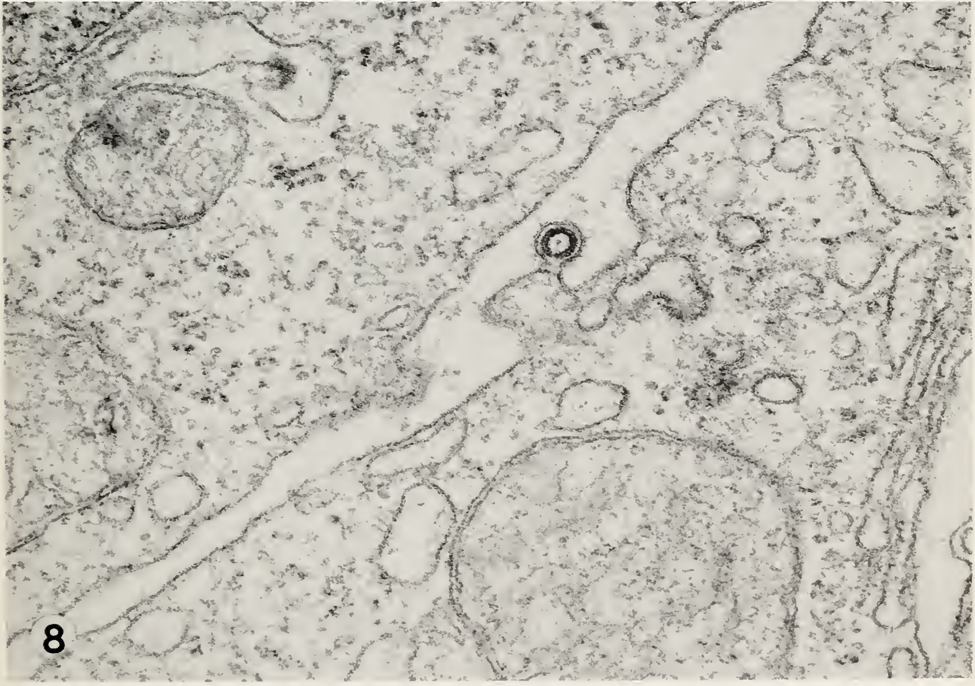
PLATE 5

FIGURE 7.—A typical erythroblast seen in a C-1A tissue culture. The high density of cytoplasmic matrix contrasts with the much clearer cytoplasm of neighboring stem cells. × 22,500

FIGURE 8.—Along the plasma membrane of a stem cell observed in a C-1A tissue culture, a leukemia virus is seen in last phase of its budding process. *Note* the continuity of plasma membrane with future viral envelope, which both show a characteristic unit membrane structure. × 63,000



7



8

PLATE 6

FIGURE 9.—Pellet prepared from filtered medium of C-1A cultures. Many typical A1 particles slightly less than 100 mμ in diameter are seen, with their characteristic electron-lucent center circled by a dense inner shell of about 50 mμ in diameter. *Arrows* point to the rare C particles. × 75,000

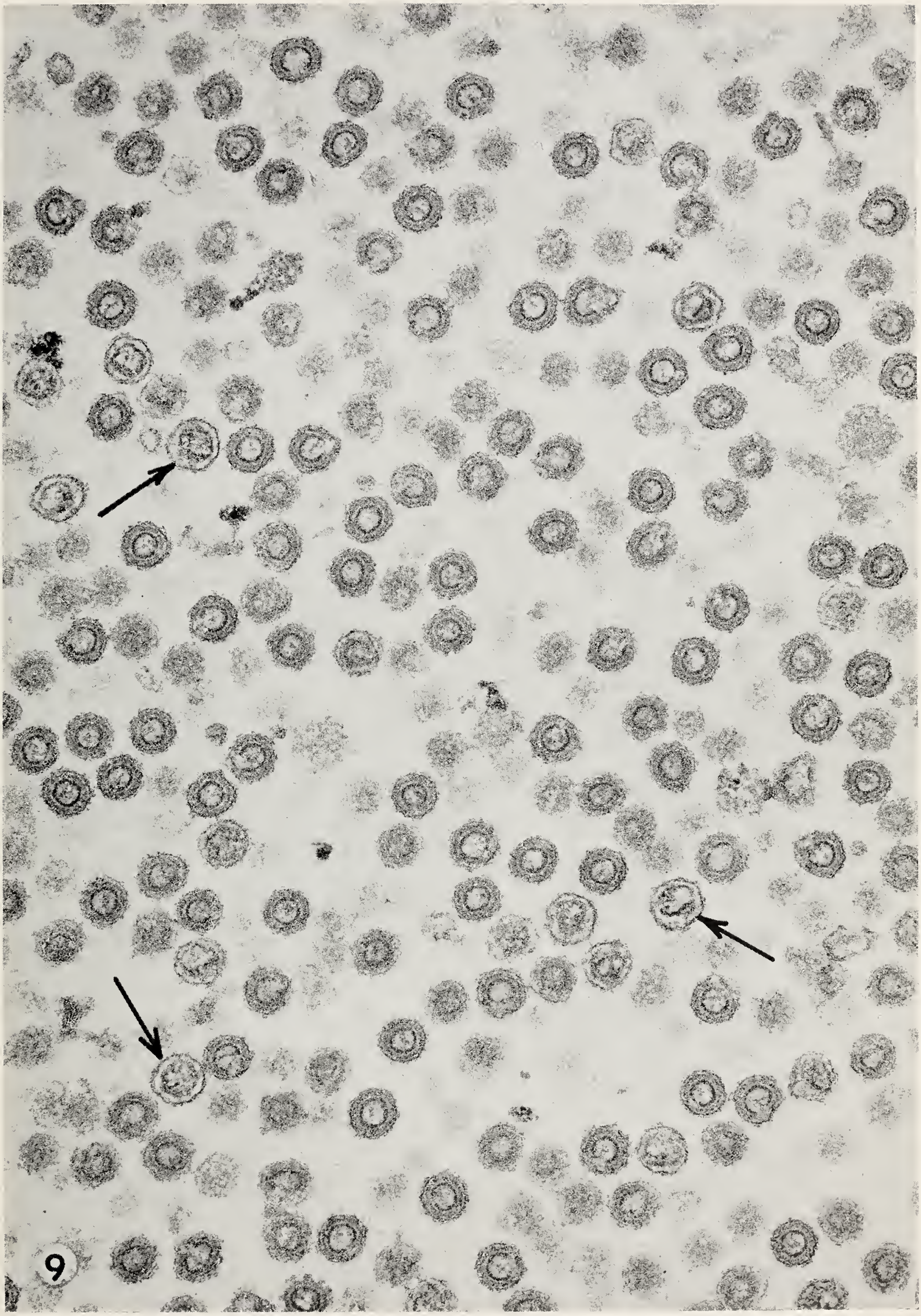
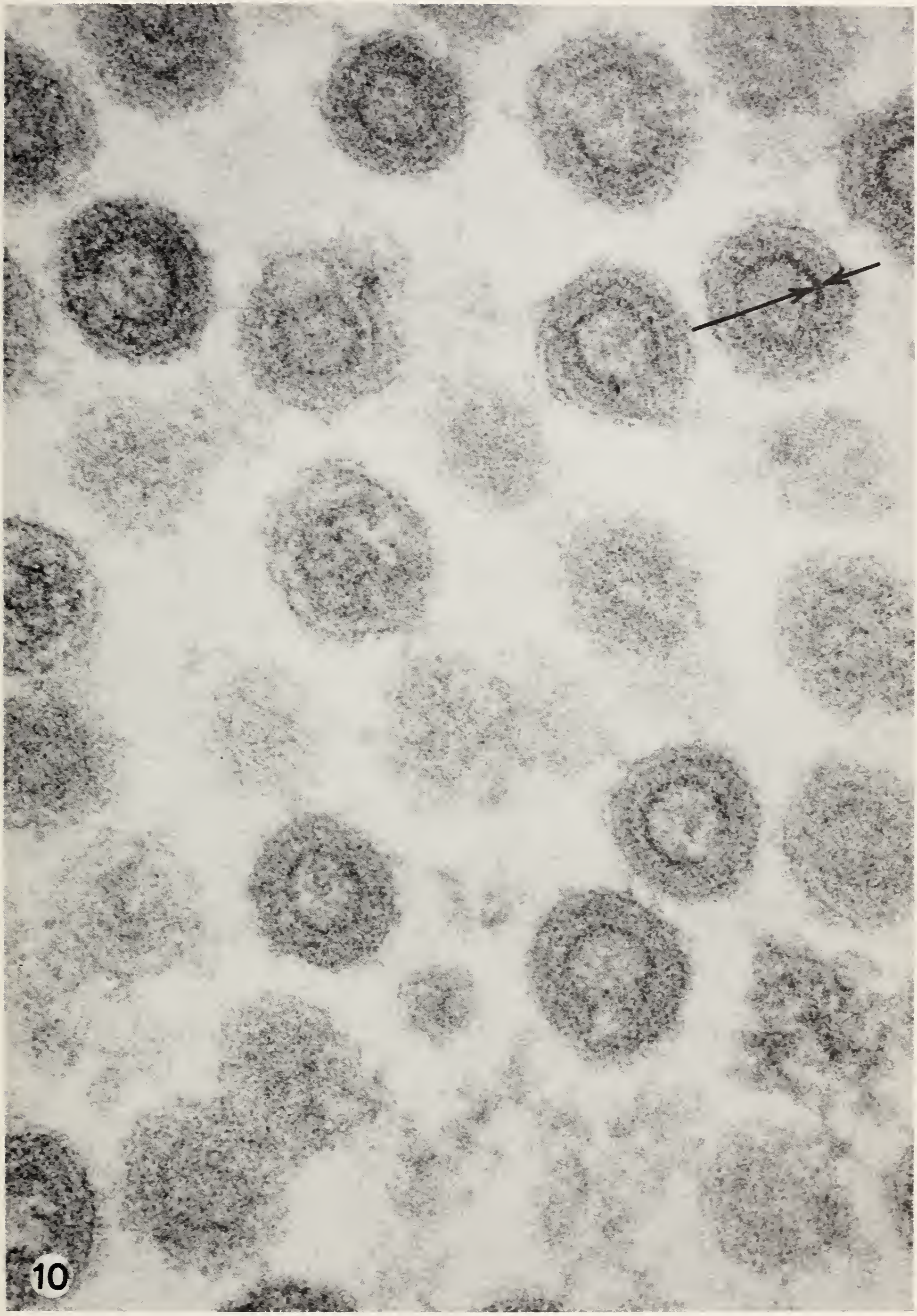

9

PLATE 7

FIGURE 10.—Pellet prepared from filtered medium from C-1A tissue culture. At this high magnification the inner shell (*arrows*) is better seen and seems always to have a maximum affinity for the "electron stains" used (uranyl and lead). This inner core seems to be approximately 50 A thick. × 260,000


10

DISCUSSION

Dr. Dmochowski: As chairman I would like to make a few remarks pertaining to this session. It may be presumptuous, and even risky, to quote or to compare anything from the field of microbiology with anything in the field of virology. Nevertheless, one is tempted to do so despite the fact that microbiology and virology apparently at first twins have now become heterozygous twins, if not siblings, competing for prestige in basic sciences and importance in applied science and clinical research.

With due respect to these competing members of a family of benevolent or malevolent agents in the life of animals and man, I would like to quote from a delightful essay by the late Dr. Woods, which is an introduction to a very fine book on the function and structure of microorganisms. He compares the microbial structure—as he calls it, edifice—to the edifice or building by Sir Christopher Wren. "Do we ask also our microbes such an element of beauty?" Even if we do not we certainly get it. Those who read the book will agree with Dr. Kenneth McQuillan that ". . . a microbial agent may be wondrous small but it is wondrous elaborate." And so is the viral agent.

The amount of knowledge gained about the structure and function of microbial and viral agents in the last 10 years is indeed impressive. One of the methods or techniques contributing greatly to our knowledge of the structure and function of the cinderella, who is now the champion agent in microbiology—that is, viruses—is electron microscopy. It can be combined with ease with such techniques as bioassay tissue culture, biochemical, biophysical, and immunological techniques.

It is, further, a common meeting ground for a virologist, a microbiologist, and now I would like to add mycoplasmologist, or one who is imbued with "mycoplasmania." And, further, it is a common meeting ground for immunologists, biochemists, biophysicists, and even—and here I speak with great reverence—molecular biologists.

It is indeed a great distance away, although apparently only 12 years ago the first sections of tissues were produced and examined under the electron microscope. This probing into the field of darkness and shadows of the intimate parts of the cell soon made them common knowledge, seldom recognized as anything of beauty, maybe because of the gradually increasing common exposure.

The explorer's mind, never satisfied, went further into the search for the unknown structure of matter, sometimes, but not always a part of cells so thoroughly if sometimes roughly searched. This led us to the knowledge of the structure and even substructure of the agents so different apparently from the older siblings (bacteria), that is, viruses.

The stage has been reached when X-ray diffraction with its latest sophisticated methods finds a common meeting ground in the exploration of the structure of viruses and, we hope, in the near future, their nucleic acids. Thus, probing into the very basis of life, electron microscopy serves as a humble tool in the search for virus particles in normal and malignant cells. It is not only a method, but a scientific discipline, and, as the latter, it is known to its disciples as a tool which also has its limitations.

We have had many interesting papers concerning this. I am sure the discussants also will be the first to point out the need for support from other scientific disciplines in our present search for the true nature of leukemia and the true part played by the viral agents in the origin of leukemia in animals and maybe in man.

Dr. Feldman: I have two comments to make relative to the previous paper. First of all, in reference to particles in control animals, we examined thymus, lymph node, spleen, bone marrow, liver, lung, and kidney from 10 normal C3Hf mice. We found particles in the thymus of 7 of 10 animals and in bone marrow in 1 of 7. Very few particles were present and particles with nucleoids appeared in the thymus of only

3 animals. In thymus, we also observed particles budding from the cell membranes of lymphocytes and lymphoblasts and from membranes of vacuoles within epithelial cells.

My second comment concerns the different cells from which particles are formed. Previously, we described particles budding from epithelial cells of salivary glands and mammary glands of leukemic C3Hf mice. More recently, we found particles, appearing to bud off from the cell membranes of pancreatic acinar cells, in the pancreas of leukemic C3Hf mice. Besides budding and doughnut-type particles and cylindrical structures, particles with nucleoids were present in intercellular spaces, occasionally in large numbers.

Dr. Masurovsky: I have a question regarding Dr. de Harven's presentation. That smaller body he observed and attributed to the lactic dehydrogenase (LDH) agent—does he have immunological or other proof that it is indeed the LDH agent?

Dr. de Harven: For LDH, yes, tests were made in Dr. Riley's laboratory, and indeed, our material induces a significant increase in the plasma level of LDH enzyme of LDH-agent-free test mice. We have not observed any recognizable mycoplasma in our electron microscope study of the C1A cell line, and biological tests have also been negative.

Chairman Dmochowski: Of course, that doesn't prove much and you understand why. Only a positive test really proves something.

Dr. de Harven: Yes, indeed, but we have another cell line that is contaminated, as shown by biological tests which are, therefore, sensitive enough in that case.

Dr. Haguenau: I would like first to comment on Dr. de Harven's beautiful pictures. I was much impressed with the ultrastructural aspect of some of his platelets, and I think that at least from the electron microscopy point of view these were not normal platelets at all and they might better be interpreted as undifferentiated platelets. They have exactly the same structure as fragments of immature megakaryocytes. In other words, normal differentiation into platelets has not occurred so that this might be an instance of malignant megakaryocytic cytoplasm, which is interesting since these cells are not thought of as being malignant in leukemia.

Secondly, I would like to ask if, in Dr. de Harven's opinion, it still holds that there is a much greater formation of virus particles in megakaryocytes rather than in the other types of cells and if they have done any quantitative type of study concerning this special point.

I ask this because it is on this assumption, the greater quantity of viruses in megakaryocytes, that we are at present very much involved in the study of megakaryocytes in human leukemia. Search for virus particles in human megakaryocytes has not been achieved at least on a great quantity of cells. With Mrs. J. Arnoult we have undertaken such a study and seen, up to now, about 400 megakaryocytes in human chronic leukemia. We never found any virus particles although "virus-*like*" particles are numerous. What I would like to know is whether it is still warranted to go on with this tedious search in megakaryocytes?

We have looked at two chronic myeloid leukemias and three chronic lymphoid leukemias. Unfortunately, we have been unable to find megakaryocytes in *acute* leukemia in which the number of these cells is very much lowered. We had to be content, therefore, with these chronic myeloid leukemias.

Search for megakaryocytes in leukemic bone marrow is strenuous work since we have to examine first with the phase contrast microscope all the blocks prepared for electron microscopy, and sometimes one has to look at as many as 30 blocks for one case to find areas in which these megakaryocytes are present.

No viruses were ever observed but "virus-like" particles may be very misleading as demonstrated by a murine leukemic megakaryocyte with typical Rauscher virus in its cytoplasm and a human megakaryocyte with what we may call virus-*looking* particles. These are specific granules. These spherical granules are very well preserved, but despite this there has been a retraction of their center so that the false impression of a "nucleoid" is created. It is sometimes very difficult to judge between viruses and virus-like particles when the specific granules are all in a row. This is a frequent finding and only one example. There are other examples of other virus-like particles such as PPLO elementary bodies but I will talk about those later.

There is another important question relative to the fact that the A1 type of particle has been found in these tissue cultures. I thought that the definition of this A1 type of particle, that is, immature or early stage of formation of C particle, was that it had, at least in the early stages, no nucleoid—the nucleoid is not really formed. Therefore, I would like to hear more on the significance of the isolation of this early type of particle and especially on its degree of infectivity.

Chairman Dmochowski: Would you mind repeating your statement pertaining to megakaryocytes in human chronic lymphatic myeloid leukemia? What did you see? Virus or virus-like particles?

Dr. de-Thé: I wish to confirm that the second slide presented by Dr. de Harven represents the LDH virus. The particles have the shape, size, and structure of the LDH virus we studied with Dr. Notkins.

I have a suggestion concerning the morphologic classification of the different virus particles seen in leukemic mice. Since the beginning of this session, particles type A, A1, C, and immature particles have been described. When one realizes that type A1, described by de Harven means immature C particles for Dalton, one can easily realize a source of confusion. Moreover Dalton described as A1 particles the intracytoplasmic particles, budding from the endoplasmic reticulum in the plasma cell tumors in mice, whereas A1 particles of de Harven represent extracellular virions. This situation, unclear for the electron microscopists, is really confusing for the uninitiated. I wish, therefore, to propose that electron microscopists meet during this Conference to clarify this situation and to propose a morphologic classification of the murine and avian RNA tumor viruses. This will help all microscopists to speak the same language and therefore to increase the value and preciseness of our field. Moreover, this will help the study of human leukemias where particles resembling those of murine and avian leukemias have been described.

Dr. Rich: We can arrange for such a meeting to be held. (A meeting attended by Drs. Dalton (Chairman), de Harven, de-Thé, Dmochowski, Feldman, Haguenau, Levy, Rich, and Zeigel was held October 14, 1965. The need for re-evaluation of the morphologic classification of murine tumor viruses was discussed and a further meeting proposed.)

Dr. Heine: Dr. de Harven, you showed electron micrographs of plasma pellets, taken at the 3d day of study, which contained virus particles and, also, much debris. Later in the experiment there was no debris in the pellets. Do you have such results often, or is the difference due to cutting through different layers of the pellets?

Dr. de Harven: We know we have "sampling problems." Unfortunately, as you know, in such material it is almost impossible to get quantitative results. Our interpretation of this indeed surprising fact would be that the pellets are probably slightly bigger in later stages and we have, therefore, a greater chance to cut through concentrations of purified viruses. But, of course, occasionally we see such contaminating debris also in later pellets.

Dr. Dutcher: I realize this is a Murine Leukemia Conference, but I just can't hesitate to interject the cow, especially after hearing some of the interesting findings of Dr. de Harven. Dr. Larkin, in our laboratory, has had a great deal of success recently in separating several different types of particles from the milk of cows with leukemia. One of the particles, which is an almost purified, homogeneous preparation, resembles very closely, morphologically and by size, the smaller particles that you have demonstrated so beautifully in your electron micrographs.

We also considered the possibility that an LDH virus might be present in these animals and tried to learn whether purified preparations of these milk concentrates, containing the aforementioned particles, when injected into mice might produce an elevation of LDH activity. We turned this material over to Dr. Riley of Sloan-Kettering and to date he has been unsuccessful in demonstrating LDH activity in inoculated mice.

We are still considering this possibility, however, and I think we also should realize that LDH agents may be species-specific and also that a whole family of LDH agents eventually may be recognized. So quite possibly, if we want to demonstrate the presence of LDH activity for a particle which may be related to this particular virus in cows, it might be more appropriate to use the cow as an assay system. Morphologically, there appears to be a great deal of similarity between the smaller particle you showed and the ones we have observed in purified preparations of leukemic cow's milk.

Morphology and Development of Some Murine Leukemia Viruses [1, 2]

Tokichi Yumoto,[3] Louis Recher,[4] John A. Sykes, *and* Leon Dmochowski, *Department of Virology, The University of Texas M. D. Anderson Hospital and Tumor Institute, and Department of Microbiology, Baylor University College of Medicine, Texas Medical Center, Houston, Texas*

THE fine structure of virus particles associated with some forms of murine leukemia was described by Dmochowski (*1*, *2*), Dmochowski *et al.* (*3*), Dalton (*4*, *5*), Dalton *et al.* (*6*), de Harven (*7*), de Harven and Friend (*8*), Graffi *et al.* (*9*), Bernhard (*10*), Amano (*11*), Parsons (*12*), Rich and Johns (*13*), Zeigel and Rauscher (*14*), and by Feldman and Gross (*15*). Virus particles were found in all the examined tissues of mice with spontaneous and induced leukemia and of rats with induced leukemia. All virus particles in tissues from animals with different types of leukemia so far investigated were found to have essentially an identical ultrastructure regardless of their source. They consisted of three membranes: a dense inner membrane, most distinct in immature virus particles and believed to transform into an electron-dense nucleoid during the process of maturation, and two outer membranes, both acquired by the process of budding from the plasma membrane or membranes of cytoplasmic vacuoles and granules. While the structure of all murine leukemia virus particles appears to be identical, slight differences in the size of virus particles present in different murine leukemias were reported by Dalton *et al.* (*6*).

Results of some of the studies on the biological effect of different murine leukemia viruses inoculated into mice with a high and low incidence of spontaneous leukemia have been reported (*16*). These studies have now been complemented by electron microscope studies of different tissues

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported in part by Public Health Service research grants CA-04140-06 and CA-05831-02 and by Public Health Service contract 43-65-585 from the National Cancer Institute, and by grant E-94-E from the American Cancer Society, Inc.

[3] Fellow of the China Medical Board of New York, Inc.

[4] Fellow of the Leukemia Society, Inc.

from high- and low-leukemia-strain mice with leukemia induced by Passage A (Gross), Moloney, Friend, or Rauscher virus and also of different tissues from low-leukemia-strain mice with leukemia after inoculation of different combinations of these viruses.

MATERIAL AND METHODS

AKR/Dm/TEX[5] and BALB/c/Dm/TEX mice, when 4 to 9 days old, were inoculated intraperitoneally with a virus preparation (Gross, Moloney, Friend, or Rauscher) in varying dilutions. These viruses were designated "original." Other preparations of these viruses after one passage in AKR mice were designated "AKR." In addition, 4- to 9-day-old BALB/c mice were inoculated 24 hours apart with 0.2 ml of the following combinations of virus preparations in 10^{-2} dilution: Gross and Friend, Gross and Rauscher, Moloney and Friend, Moloney and Rauscher, Moloney and Gross, and Friend and Rauscher.

Twenty AKR/Dm/TEX high-leukemia-strain mice and 29 BALB/c/-Dm/TEX low-leukemia-strain mice with leukemia induced by the different mouse leukemia viruses—Passage A (Gross), Moloney, Friend, and Rauscher—were used in the present study. In addition, 4 C3Hf/Gr/Dm low-leukemia-strain mice with leukemia following inoculation with Passage A virus were used. The leukemic mice used in the present study varied in age from 1 month to 15 months.

Of the 20 leukemic AKR mice, 7 had been inoculated with either Passage A, Moloney, Rauscher, or Friend virus, and 13 with the same viruses after one passage in AKR mice.

Of 13 leukemic BALB/c mice, 10 were inoculated with either Passage A, Moloney, or Friend virus and 3 with Passage A, Friend, or Rauscher virus.

Sixteen leukemic BALB/c mice were inoculated with the following combinations of viruses given 24 hours apart: Gross and Friend, Gross and Rauscher, Moloney and Friend, Moloney and Rauscher, Moloney and Gross, and Friend and Rauscher.

In addition, organs from 3 mice, found nonleukemic on gross and histologic examination, were studied in the electron microscope. Included in this group were 1 AKR mouse inoculated with Friend virus after passage in AKR mice and 2 BALB/c mice, 1 of which was inoculated with Passage A and Rauscher viruses and the other with Moloney and Passage A viruses.

Whenever possible, the following tissues from the leukemic mice were taken for histologic and electron microscopic examination: thymus, lymph nodes, bone marrow, spleen, and liver.

---

[5] Obtained from Texas Inbred Mice Co., 305 Almeda-Genoa Road, Houston, Texas.

Virus strains.—Passage A (Gross) virus, originally received from Dr. L. Gross, was passaged in our laboratory in C3Hf/Gr/Dm mice. The term "Passage A (Original) virus" has been introduced to identify Gross virus preparations obtained from C3Hf/Gr/Dm mice with leukemia. The term "Passage A (AKR) virus" has been used to identify virus obtained from AKR/Dm/TEX mice with leukemia induced by Passage A (Original) virus.

Moloney virus, received from Dr. J. B. Moloney, was passaged in BALB/c/Dm/TEX mice. The term "Moloney (Original) virus" has been used to designate virus preparations derived from leukemic tissues of BALB/c/Dm/TEX mice. The term "Moloney (AKR) virus" has been used for virus preparations from leukemic organs of AKR/Dm/-TEX mice inoculated with Moloney (Original) virus preparations.

Friend virus, obtained from Dr. Charlotte Friend, was passaged in BALB/c/Dm/TEX mice. The term "Friend (Original) virus" denotes virus preparations derived from leukemic tissues of BALB/c/Dm/TEX mice. The term "Friend (AKR) virus" denotes virus preparations from leukemic AKR/Dm/TEX mice inoculated with Friend (Original) virus.

Mice were killed and autopsied when symptoms of enlargement of the thymic gland, spleen, liver, or peripheral lymph nodes were detected. Tissue samples of thymus, spleen, liver, lymph nodes, and bone marrow were removed from leukemic animals for histologic and electron microscopic examination. The tissues selected for electron microscopy were cut into small pieces and fixed for 18 hours at 4° C in 2% osmic acid buffered with sodium and potassium phosphate (*p*H 7.2) with added glucose, according to the method of Millonig (*17*). The tissues were dehydrated in a series of graded ethanols (30–100%) at 30-minute intervals. They were then brought through a mixture of absolute alcohol and *n*-butylmethacrylate (equal volumes) for 1 hour, followed by two changes of methacrylate for 1 hour each, prepolymerized monomer overnight, and polymerized in the methacrylate with 1% benzoyl peroxide at 56° C for 24 hours. Sections were cut with glass knives on a Porter-Blum (Servall) automatic microtome and stained with a saturated solution of uranyl acetate for 2 hours, washed in triple distilled water, and then counterstained with a solution of lead tartrate for 10 minutes (*18*). After staining, the sections were coated with a thin film of carbon and examined in an RCA EMU 3E or 3G electron microscope at 80 or 100 kv. Original magnifications of the electron micrographs varied from 7,000 to 22,000 diameters.

RESULTS

The material examined by electron microscopy is presented in tables 1 and 2. The histopathologic diagnosis was made by light microscopic examination of the same organs, parts of which were taken for electron microscope examination. It is of interest that some AKR mice and

TABLE 1.—Material examined by light and electron microscopy

Strain of virus	Strain of mice inoculated	Source of virus, strain of mice	Dilution of virus preparation	Number of mouse	Survival time (days)	Pathologic diagnosis
Passage A(Gross)	AKR	C3H/f	10^{-4}	1	340	Lymphatic leukemia
			10^{-1}	2	460	" "
		AKR	10^{-2}	1	183	Lymphatic leukemia
			10^{-3}	2	197	" "
			10^{-4}	3	212	" "
	BALB/c	AKR	10^{-3}	1	397	Lymphatic leukemia
	C3H/f	C3H/f	Undiluted	1	85	Lymphatic leukemia
			"	2	85	" "
			"	3	85	" "
			"	4	85	" "
Moloney	AKR	BALB/c	10^{-3}	1	80	Lymphatic leukemia
		AKR	10^{-3}	1	242	Lymphatic leukemia
			10^{-4}	2	240	" "
			10^{-4}	3	260	" "
	BALB/c	BALB/c	10^{-2}	1	89	Lymphatic leukemia
Friend	AKR	BALB/c	10^{-2}	1	30	Erythroid leukemia
			10^{-2}	2	30	" "
			10^{-2}	3	30	" "
			10^{-2}	4	41	" "
		AKR	10^{-4}	1	277	No leukemia
			10^{-4}	2	257	Lymphatic leukemia
			10^{-4}	3	267	" "
			10^{-3}	4	301	" "

Friend	BALB/c	BALB/c	10^{-3}	1	41	Erythroid leukemia
			10^{-3}	2	110	“ “
			10^{-4}	3	139	Lymphatic leukemia
			10^{-3}	4	29	Erythroid leukemia
		AKR	10^{-3}	1	383	Lymphatic leukemia
Rauscher	AKR	AKR	10^{-3}	1	219	Lymphatic leukemia
			10^{-3}	2	219	“ “
			10^{-3}	3	219	“ “
			10^{-1}	4	159	“ “
	BALB/c	BALB/c	10^{-3}	1	146	Erythroid leukemia
			10^{-2}	2	35	“ “
			10^{-2}	3	39	“ “
			10^{-2}	4	41	“ “
			10^{-2}	5	41	“ “
		AKR	10^{-1}	1	75	Erythroid leukemia

TABLE 2.—Interference* studies in BALB/c mice

Virus strains (Original)	Number of mouse	Survival time (days)	Pathologic diagnosis
Passage A and Friend	1	40	Erythroid leukemia
	2	35	" "
	3	35	" "
Passage A and Rauscher	1	39	Erythroid leukemia
	2	39	No leukemia
	3	50	Erythroid leukemia
Moloney and Friend	1	33	Erythroid leukemia
	2	33	" "
	3	33	" "
Moloney and Rauscher	1	109	Lymphatic leukemia
	2	100	" "
	3	120	" "
Moloney and Passage A	1	42	No leukemia
	2	81	Lymphatic leukemia
	3	105	" "
Friend and Rauscher	1	34	Erythroid leukemia
	2	34	" "
	3	34	" "

*The second virus preparation was inoculated 24 hours after inoculation of the first virus preparation.

BALB/c mice, which had been inoculated with Friend or Rauscher virus preparations obtained from AKR mice with erythroid leukemia induced by either of the two viruses, did not develop erythroid leukemia but developed lymphatic leukemia after a long latency period varying from 159 to 383 days.

Virus particles of the murine leukemia type were found in almost every tissue sample examined. Although 4 different murine leukemia viruses—Passage A, Moloney, Friend, or Rauscher—were inoculated into 3 strains of mice—AKR/Dm/TEX, BALB/c/Dm/TEX, and C3Hf/Gr/Dm—no essential difference in the ultrastructural morphology and mode and sites of development of the virus particles was noticed in leukemic organs from mice of the different strains, irrespective of the type of leukemia examined in the electron microscope. Most virus particles were spherical or spheroid and showed the typical structure of murine leukemia viruses. Frequently, the virus particles were found in cytoplasmic inclusions. Occasionally, cylindrical forms and particles with tails were observed. In a few instances, virus particles different from murine leukemia virus particles were found. They were composed of two concentric membranes usually of equal electron density with an electron-lucent center, which has been described by others in plasma cell tumors (*4*, *19*), transplantable lymphomas of Swiss mice (*20*), and in spontaneous mammary tumors of CBA mice (*21*, *22*).

Differentiation of murine leukemia viruses on the basis of their ultrastructure was virtually impossible. However, differentiation of the two histopathological types of leukemias—lymphatic and erythroid—was possible even in sections examined in the electron microscope.

Lymphatic Leukemia

In lymphatic leukemia, virus particles were produced mainly by two types of cells: lymphoid cells and megakaryocytes. In lymphoid cells, the particles budded from the plasma membrane (figs. 1a and b), and in the megakaryocytes they developed within cytoplasmic channels (fig. 2). They occasionally budded from the membrane of specific granules (fig. 3). Virus particles were occasionally seen in the granules of neutrophils and in vacuoles of endothelial cells of blood vessels and of epithelial cells of the thymus gland (fig. 5b). Immature particles were mostly spherical and uniform in size. The immature particles showed a dense inner membrane with an electron-lucent center sometimes referred to as a "ring-shaped nucleoid" (Dalton) with an outer membrane and, in many particles, with a distinct intermediate membrane (figs. 4a and b). Some immature virus particles were linked together by their outer membranes forming a short chain (fig. 5a).

In sections of bone marrow, spleen, and liver, cylindrical or tubular forms, composed of the structure resembling the structure of immature particles, were found in megakaryocytes and occasionally in the intercellular spaces (figs. 6a and b). Dumbbell forms with the structure of immature particles were also found along cell surfaces (figs. 7a and b). Mature particles, often in large numbers, were found in intercellular spaces and within vacuoles of macrophages (figs. 8a, b, and c) and reticuloendothelial cells. These particles showed a double outer membrane with a centrally located electron-dense nucleoid of variable structure.

Mature virus particles could be divided into two categories:

1) Particles with an electron-dense nucleoid and a fine reticular inner structure, surrounded by an outer double membrane, were seen. The shape and size of the outer double membrane varied markedly. Giant particles showing marked pleomorphism were occasionally seen (figs. 9a, b, c, d). These particles will be referred to as C-1 particles.

2) Particles with a less electron-dense nucleoid surrounded by a double membrane somewhat smaller and of more uniform size than those described as C-1 were also seen. These particles appeared to represent a transitional stage in the development of immature particles to mature particles (figs. 10a and b). These particles will be referred to as C-2 particles.

Mature particles with a dense nucleoid (C-1 particles) were more frequently observed in lymphatic leukemia than in erythroid leukemia. Only mature virus particles with tail-like structures were occasionally observed in lymphatic leukemia (figs. 11a, b, and c).

Immature and mature virus particles were most numerous in sections of mesenteric lymph nodes, spleen, thymus, and bone marrow (figs. 12a, b, and c). In the intercellular spaces, virus particles were found both singly and in aggregates. When in aggregates, they were sometimes embedded in an electron-dense, amorphous substance. In macrophages and reticuloendothelial cells, the virus particles were usually seen in large numbers within vacuoles and inclusions. Budding viruses were never found in these cells. Macrophages often showed degenerative changes of the cytoplasm and nucleus and in some instances contained viral and nonviral inclusions (figs. 13a, b, and c).

In one BALB/c mouse with lymphatic leukemia induced by Moloney virus, virus particles were observed budding from the plasma membrane of cells of the erythroid series (fig. 14). In a few instances, virus particles composed of two membranes resembling A particles were found within the endoplasmic reticulum of cells believed to be leukemic cells from cases of lymphatic leukemia in BALB/c mice (fig. 15).

In one AKR mouse with lymphatic leukemia inoculated with Friend virus, aggregates of particles with two concentric membranes were observed in the cytoplasm of plasma cells in sections of a mesenteric lymph node (figs. 16 a, b). The outer membrane had a diameter of 753 A, and the inner membrane a diameter of 525 A. In sections of the same tissue, virus particles were also seen budding from the membranes of the endoplasmic reticulum of some plasma cells (fig. 17).

Leukemic organs of C3H/f mice inoculated with Passage A (Gross) virus resembled leukemic organs of AKR mice with lymphatic leukemia.

Erythroid Leukemia

In this type of leukemia, the virus particles were found mainly in cells of the erythroid series (fig. 18) and in megakaryocytes. Virus particles budded from the plasma membrane and from membranes of cytoplasmic vacuoles of these cells (fig. 19). Virus particles were also seen to bud from the plasma membranes of reticuloendothelial cells, particularly Kupffer cells, and of liver cells into the Disse space in sections of the liver (figs. 20a, b, c, d, and e).

Immature virus particles had a dense inner membrane enclosing an electron-lucent center and a less dense outer membrane, and most particles also had an intermediate membrane. The budding and immature particles found in erythroid leukemia were similar in structure to those found in lymphatic leukemia. Dumbbell forms and chainlets of immature particles were occasionally observed, but cylindrical or tubular forms have, so far, not been observed.

Mature virus particles were usually spherical and of uniform size. Mature particles could also be divided into two categories already described in lymphatic leukemia. Particles of the C-2 type were more frequent in erythroid leukemia than those of the C-1 type (fig. 21). Particles with "tails" were only found in organs of leukemic mice inoculated with Rauscher virus (fig. 22).

Large numbers of virus particles were seen in the spleen and liver, organs known to be frequently infiltrated by leukemic cells. Small numbers of particles and, in some instances, no particles were found in sections of lymph nodes and thymus. Interestingly, the virus particles were never seen in large aggregates; the largest number in any one cell varied between 15 and 20. Most virus particles were seen in the intercellular spaces and in the cytoplasmic vacuoles.

Cytoplasmic inclusions in macrophages and in reticuloendothelial cells, which contained groups of virus particles, often were nonviral cytoplasmic inclusions associated with the endoplasmic reticulum (figs. 23a and b).

In addition to the particles characteristic of murine leukemia virus, particles with two membranes resembling A particles were found in the endoplasmic reticulum of leukemic cells but only in the spleen, lymph nodes, and bone marrows of leukemic BALB/c mice (fig. 24).

Interference Tests

The findings of the interference tests are summarized in table 3.

In BALB/c mice with lymphatic leukemia inoculated with Moloney and Rauscher virus or Moloney and Passage A (Gross) virus, the mode of development of virus particles, their numbers, and the occurrence of dumbbell, cylindrical, and tail-like structures were similar to those already described for lymphatic leukemia. Mice, inoculated with Passage A (Gross) and Friend virus or Moloney and Friend virus, developed erythroid leukemia. In organs of these mice, virus particles were seen budding from the plasma membranes of lymphoid, erythroid, and reticuloendothelial cells. Mice inoculated with Passage A (Gross) and Rauscher virus or Moloney and Friend virus developed erythroid leukemia. In organs of these mice, the mature virus particles observed were primarily of the C-1 type. Mice developing erythroid leukemia after inoculation of Friend and Rauscher virus showed in their leukemic organs a submicroscopic picture similar to that seen in organs of mice with erythroid leukemia induced by either of the two viruses.

DISCUSSION

As shown in table 1, AKR/Dm/TEX and BALB/c/Dm/TEX mice inoculated with Friend (AKR) or Rauscher (AKR) virus developed lymphatic leukemia but not erythroid leukemia, as one would probably expect. The source of virus in these experiments was leukemic organs of AKR mice with erythroid leukemia induced by either Friend (Original) or Rauscher (Original) virus. It is therefore believed that the Gross virus, known to induce spontaneous leukemia in the AKR mice, was responsible for the development of lymphatic leukemia in these mice (*16*).

TABLE 3.—Relationship of histologic diagnosis to types and sites of virus particles

Strain of virus*	Histologic diagnosis	Budding†				Types of mature virus particles†				Cyto-plasmic inclusion	Immature virus particles†	
		Lympho-cytes	Erythro-cytes	Mega-karyo-cytes	Reticulo-endothe-lial cells	Pleomor-phic form	Tails	C-1> C-2	C-1< C-2		Cylin-drical	Dumb-bell
PA + Fr	Erythroid leukemia	+	+	+	+	—	—		+	+	—	+
PA + Ra	" "	—	+	+	+	+	+		+	+	—	+
Mo + Fr	" "	+	+	+	+	+	—		+	+	—	+
Mo + Ra	Lymphatic leukemia	+	—	+	—	+	—	+		+	—	—
Mo + PA	" "	+	—	+	—	+	—	+		+	+	+
Fr + Ra	Erythroid leukemia	—	+	+	+	—	—		+	+	—	+
	Lymphatic leukemia	+	+	+	—	+	+	+		+	+	+
	Erythroid leukemia	—	+	+	+	—	—		+	+	—	+

*PA, Passage A; Fr, Friend; Ra, Rauscher; Mo, Moloney.

†+ denotes presence of virus particles (immature or mature) or sites of virus particle development; — denotes absence of virus particles (immature or mature) or sites of virus particle development.

In the present study, virus particles have been found in the organs of all leukemic AKR, BALB/c, and C3H/f mice examined. In addition, virus particles have been found in the organs of nonleukemic AKR mice inoculated with Friend virus and in those of BALB/c mice inoculated with Moloney and Passage A (Gross) or Passage A (Gross) and Rauscher viruses. Minor differences in the distribution of virus particles have been observed in lymphatic and erythroid leukemia. Leukemic cells have been the main sites of production of virus particles. Therefore, it was not surprising that, in lymphatic leukemia, large numbers of virus particles have been found in the thymus, lymph nodes, and spleen—organs known to contain extensive leukemic infiltrates.

Virus particles have been seen to develop by budding from the plasma membranes of leukemic cells and membranes of cytoplasmic channels of megakaryocytes. They have been found released in the intercellular spaces and in vacuoles of macrophages and of reticuloendothelial cells. It is of interest that mature virus particles have occasionally been observed in the granules of neutrophils, cytoplasmic inclusions, and vacuoles in Kupffer cells, in endothelial cells, and in epithelial cells of the thymus. Budding of virus particles from the plasma membrane of Kupffer and reticulum cells has also been observed in the present study. Budding from the plasma membrane of liver cells into the Disse space has also been seen. Dmochowski *et al.* have reported budding of virus particles from microvilli of liver cells (*23*) and from plasma membrane of eosinophils (*3*). Feldman *et al.* observed budding of plasma membrane of epithelial cells of the salivary gland (*15*) and of epithelial cells of mammary glands during pregnancy (*24*). It appears therefore that the murine leukemia virus can develop in at least some normal cells, as has previously been reported for megakaryocytes (*3*, *6*). Bernhard (*10*) and Dalton (*4*) classified particles associated with tumors on the basis of their morphology into 6 groups: A1, A2, A3, B, C, and D. Type C particles were commonly observed to bud from the plasma membrane of leukemic cells and from the membranes of cytoplasmic channels of megakaryocytes. The mature type C particles have a centrally located, electron-dense nucleoid and double outer membrane. This type of particle has been shown to be associated with leukemia of Gross (*1*, *12*, *25*, *26*), Moloney (*6*), Friend (*8*), Rauscher (*14*), Graffi (*9*), and with SL mouse leukemia (*11*), as well as with spontaneous leukemia of AKR (*27*) and C3Hf/FgLw mice (*6*) and with radiation-induced leukemia of C3HBi and RF mice (*28*, *29*).

Most virus particles observed in the present study were of type C. They could be subdivided into type C-1 and type C-2 particles. Type C-1 are mature particles with an electron-dense nucleoid; type C-2 particles have a less dense nucleoid resembling somewhat immature forms. This subdivision into two groups is somewhat significant since it was found that type C-1 particles seemed more frequently associated with lymphatic leukemia and type C-2 particles more frequently associated with erythroid leukemia.

It is of interest that unusual forms of virus particles, such as cylindrical and pleomorphic, were observed only in lymphatic leukemia. The mouse strain did not appear to influence these findings. Dmochowski *et al.* (*3*), Feldman and Gross (*15*), and Dalton *et al.* (*30*) have reported that the cylindrical or tubular forms of virus may possibly segment to form the doughnut-type particles. It appears that the dumbbell forms of virus particles subdivide to form the doughnut-type particles.

Virus particles with an ultrastructure different from that of the murine leukemia type observed in this study closely resembled particles described by Dalton (*4*), de Harven and Friend (*20*), and Recher *et al.* (*21*). These particles, resembling immature Bittner virus particles, have been found in the present study in leukemic cells, plasma cells, and reticuloendothelial cells of lymph nodes, thymus, and spleen. Virus particles with a similar structure were shown to occur in mice with Moloney leukemia (*6*), Lymphoma 6C3HED (*31*), Ehrlich ascites (*4*), mouse ascites tumors E.L.4 and C1498 (*32*), and in Sarcoma 37 and Sarcoma 61 (*4*). Similar particles have also been observed in sections of lymph nodes of healthy BALB/c and DBA/2 mice by Dr. Gabriel Seman in our laboratory (unpublished data).

These immature virus particles resembling immature Bittner virus have been observed in plasma cells of mesenteric lymph nodes of a single AKR mouse with lymphatic leukemia after inoculation of Friend (AKR) virus. They have also been found in mammary tumors of mice of various strains (*1*, *10*, *22*), in a spontaneous leukemia of a Swiss mouse (*20*), and in primary and transplanted plasma cell tumors (*19*). This last group is of interest with reference to the case observed in this study, since these particles have been observed by us in plasma cells only. These particles therefore might be identical with those described by Dalton *et al.* (*19*). Dalton (*4*) reported that these particles were found in malignant cells only, but the findings presented here suggest that identical particles may also occur in normal plasma cells.

Dmochowski *et al.* (*33*) reported structures resembling mycoplasma in thin sections of lymph nodes from patients with leukemia and lymphoma and in thin sections of plasma pellets obtained from leukemic mice and from leukemic patients. The ultrastructure of some forms of mycoplasma has been described by van Iterson and Ruys (*34*), Edwards and Fogh (*35*), Domermuth *et al.* (*36*), Anderson and Barile (*37*), Hummeler *et al.* (*38*, *39*), and Klieneberger-Nobel (*40*). Hummeler *et al.* (*38*, *39*) stated that the ultrastructure of mycoplasma was variable and that some forms (elementary bodies) of mycoplasma are similar in morphology to murine leukemia virus particles. It is occasionally very difficult to differentiate virus particles from the developmental or elementary bodies of mycoplasma. Some forms of mycoplasma apparently also resemble some of the cellular submicroscopic components, such as lysosomes, inclusion bodies, and secretory granules.

In the present study, electron microscopic examination of sections of various organs from leukemic AKR and BALB/c mice has revealed the presence of structures resembling mycoplasma in various organs of 20 of 53 mice studied. The findings pertaining to structures resembling mycoplasma are summarized in tables 4 and 5. These structures have been more frequently seen among and within leukemic cells and within reticuloendothelial cells, particularly macrophages. The submicroscopic morphology of these mycoplasma-like structures appears variable (figs. 25a, b, c, and d and 26a and b). These structures are, in many instances, similar to those described in electron microscope studies of mycoplasma from pure cultures (*34–39*, *41*). In addition, large vesicular structures containing many small bodies have been observed in leukemic cells and in reticuloendothelial cells (fig. 27). These bodies resemble the multivesicular bodies described in oocytes and in mature and fertilized ova by Sotelo and Porter (*42*) and present in many different cell types (*43–45*). The significance of these

Table 4.—Leukemic mice with structures resembling mycoplasma

Virus used for induction of leukemia	Number of mice examined	Number of leukemic mice with structures resembling mycoplasma		
		AKR	BALB/c	C3H/f
Passage A (Gross)	10	2	0	1
Moloney	5	2	1	—
Friend	12	2	3	—
Rauscher	10	2	2	—
Passage A (Gross) and Friend	3	—	1	—
Passage A (Gross) and Rauscher	2	—	0	—
Moloney and Friend	3	—	1	—
Moloney and Rauscher	3	—	1	—
Moloney and Passage A (Gross)	2	—	1	—
Friend and Rauscher	3	—	1	—
Total	53	8	11	1

Table 5.—Leukemic mice with structures resembling mycoplasma in various organs

Virus used for induction of leukemia	Number of leukemic mice with structures resembling mycoplasma in various organs				
	Spleen	Lymph nodes	Liver	Thymus	Bone marrow
Passage A (Gross)	2/10*	2/10	1/10	1/10	1/10
Moloney	3/5	1/5	0/5	1/5	0/5
Friend	4/12	1/12	1/12	0/12	0/12
Raucher	3/10	1/10	1/10	0/10	0/10
Passage A (Gross) and Friend	0/3	0/3	1/3	0/3	0/3
Passage A (Gross) and Rauscher	0/2	0/2	0/2	0/2	0/2
Moloney and Friend	0/3	0/3	1/3	0/3	0/3
Moloney and Rauscher	0/3	1/3	0/3	0/3	0/3
Moloney and Passage A (Gross)	1/2	0/2	0/2	0/2	0/2
Friend and Rauscher	0/3	0/3	1/3	0/3	0/3
Total	13/53	6/53	6/53	2/53	1/53

**Numerators* denote number of mice with structures resembling mycoplasma present in different organs. *Denominators* denote number of mice examined.

structures is still unknown. Sotelo and Porter concluded that these structures occur normally as a constant component in animal cells. They stated also that multivesicular bodies are self-reproducing units and that in maturity liberate small vesicles characterized by the constancy of their size and their close association (*42*). Similar structures have recently been observed and described as mycoplasma-like bodies by Seman and Dmochowski (*46*) in 7-day embryos from mice of strains prone to leukemia. It is of interest that the characteristic murine leukemia virus particles have been observed within these bodies (fig. 28). These bodies could be interpreted as being mycoplasma-like structures, but they could also be interpreted as being some normal cellular components of unknown significance. Further studies are being carried out on submicroscopic changes in cells of various organs during the course of leukemia in mice and humans.

SUMMARY

A comparative electron microscope study was carried out on ultrathin sections of bone marrow, spleen, lymph nodes, thymus, and liver of leukemic AKR/Dm/TEX and BALB/c/Dm/TEX mice inoculated with Passage A (Gross), Moloney, Friend, or Rauscher murine leukemia viruses. In all leukemic organs of mice of both strains, regardless of the type of virus inoculated, mature and immature (doughnut-type) virus particles were seen. Virus particles were more numerous in spleens of Friend and Rauscher virus-infected mice and in spleens and mesenteric lymph nodes of Gross and Moloney virus-infected animals than in other involved organs. Virus particles were frequently seen in the intercellular spaces of leukemic organs and developing in megakaryocytes and in leukemic cells. They were occasionally seen developing in plasma cells, neutrophils, erythroblasts, and, in a few instances, in parenchymatous and reticuloendothelial cells of the liver. The development of virus particles was characterized by budding from plasma membranes. Structures resembling mycoplasma were frequently found in the intercellular spaces and within cytoplasm of leukemic and reticulum cells, often in close association with virus particles. Studies were also carried out on leukemic organs from BALB/c mice inoculated 24 hours apart with any 2 of the 4 viruses. The findings in the organs of these mice were similar to those in organs of mice inoculated with any single virus. The number, size, and sites of development of the virus particles, whether Passage A, Moloney, Friend, or Rauscher, were essentially the same in the various leukemic organs. There appears to be a close morphologic similarity between the 4 leukemia viruses. However, the present studies have demonstrated that some differences apparently exist in the mode of development and some variation in the appearance of mature virus particles (types C-1 and C-2) in lymphatic and erythroid leukemia.

REFERENCES

(*1*) Dmochowski, L.: Viruses and tumors in the light of electron microscope studies. A review. Cancer Res 20: 977–1015, 1960.

(*2*) ———: The electron microscopic view of virus-host relationship in neoplasia. *In* Progress in Experimental Tumor Research (Homburger, F., ed.). New York and Basel, Karger, vol 3, 1963, pp 35–147.

(*3*) Dmochowski, L., Padgett, F., and Gross, L.: An electron microscope study of rat leukemia induced with mouse leukemia virus (Gross). Cancer Res 24: 869–899, 1964.

(*4*) Dalton, A. J.: Micromorphology of murine tumor virus and affected cells. Fed Proc 21: 936–941, 1962.

(*5*) ———: The Moloney agent. *In* Tumors Induced by Viruses. Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York and London, Academic Press Inc., 1962, pp 207–218.

(*6*) Dalton, A. J., Law, L. W., Moloney, J. B., and Manaker, R. A.: An electron microscopic study of a series of murine lymphoid neoplasms. J Nat Cancer Inst 27: 747–791, 1961.

(*7*) de Harven, E.: Ultrastructural studies on three different types of mouse leukemia. A review. *In* Tumors Induced by Viruses. Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York and London, Academic Press Inc., 1962, pp 183–206.

(*8*) de Harven, E., and Friend, C.: Further electron microscope studies of a mouse leukemia induced by cell-free filtrates. J Biophys Biochem Cytol 7: 747–752, 1960.

(*9*) Graffi, A., Heine, U., Helmcke, J. G., Bierwolf, D., and Randt, A.: Ueber den electronenmikroskopischen Nachweis von Viruspartikeln bei der myeloischen Leukaemie der Maus nach Injektion zellfreier Tumorfiltrate. Klin Wschr 38: 254–262, 1960.

(*10*) Bernhard, W.: Electron microscopy of tumor cells and tumor viruses: A review. Cancer Res 18: 491–509, 1958.

(*11*) Amano, S.: Host-virus interrelationships in cancerogenesis as observed under the electron microscope. *In* Progress in Experimental Tumor Research (Homburger, F., ed.). New York and Basel, Karger, vol 2, 1961, pp 36–66.

(*12*) Parsons, D. F.: Structure of the Gross leukemia virus. J Nat Cancer Inst 30: 569–583, 1963.

(*13*) Rich, M. A., and Johns, L. W., Jr.: Morphology of an agent associated with a murine leukemia. Virology 20: 373–376, 1963.

(*14*) Zeigel, R. F., and Rauscher, F. J.: Electron microscopic and bioassay studies on a murine leukemia virus (Rauscher): Preliminary report. J Nat Cancer Inst 30: 207–219, 1963.

(*15*) Feldman, D. G., and Gross, L.: Electron-microscopic study of the mouse leukemia virus (Gross), and of tissues from mice with virus-induced leukemia. Cancer Res 24: 1760–1783, 1964.

(*16*) Dmochowski, L., Tanaka, T., Yumoto, T., Sykes, J. A., Young, L., and Recher, L.: Studies on the biological relationship of some murine leukemia viruses. Cancer Res. In press.

(*17*) Millonig, G.: Advantages of a phosphate buffer for OsO_4 solution in fixation. Proc 19th Ann Meeting Electron Microscope Soc Amer, 1961, pp. 15–16.

(*18*) ———: A modified procedure for lead staining of thin sections. J Biophys Biochem Cytol 11: 736–739, 1961.

(*19*) Dalton, A. J., Potter, M., and Merwin, R. M.: Some ultrastructural characteristics of a series of primary and transplanted plasma-cell tumors of the mouse. J Nat Cancer Inst 26: 1221–1267, 1961.

(20) de Harven, E., and Friend, C.: Electron microscopy of Swiss mouse leukemia virus. Nat Cancer Inst Monogr 4: 291–311, 1960.

(21) Recher, L., Langford, P. A., and Dmochowski, L.: Studies on the development of Bittner virus in spontaneous mammary tumors of CBA strain mice. In preparation.

(22) Moore, D. H.: The milk agent. *In* Tumors Induced by Viruses. Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York and London, Academic Press Inc., 1962, pp 113–150.

(23) Dmochowski, L., Rogers, T., Liebelt, A. G., Liebelt, R. A., and Sykes, J. A.: Studies on leukemia in mice associated with a transplantable methylcholanthrene-induced hepatoma. Nat Cancer Inst Monogr 22: 223–236, 1966.

(24) Feldman, D. G., Gross, L., and Dreyfuss, Y.: Electron microscopic study of the Passage A mouse leukemia virus in mammary glands of pregnant, virus-injected, C3H(f) mice. Cancer Res 23: 1604–1607, 1963.

(25) Bernhard, W., and Gross, L.: Présence de particules d'aspect viral dans les tissus tumoreux de souris atteintes de leucémies induites. C R Acad Sci 247: 160–163, 1959.

(26) Bernhard, W., and Guerin, M.: Présence de particules d'aspect virusal dans les tissus tumoreux de souris atteintes de leucémies spontanées. C R Acad Sci 247: 1802–1805, 1958.

(27) Dmochowski, L., and Grey, C. E.: Subcellular structures of possible viral origin in some mammalian tumors. Ann NY Acad Sci 68: 559–615, 1957.

(28) Dmochowski, L., Grey, C. E., and Gross, L.: The role of viruses in x-ray induced leukemia. 12th Symposium on Fundamental Cancer Research, Houston, 1958. Austin, Univ Texas Press, 1959, pp 382–399.

(29) Parsons, D. F., Upton, A. C., Bender, M. A., Jenkins, V. K., Nelson, E. S., and Johnson, R. R.: Electron microscopic observations on primary and serially passaged radiation-induced myeloid leukemias of the RF mouse. Cancer Res 22: 728–736, 1962.

(30) Dalton, A. J., Haguenau, F., and Moloney, J. B.: Further electron microscopic studies on the morphology of the Moloney agent. J Nat Cancer Inst 33: 255–275, 1964.

(31) Sobin, L. H.: Virus-like particles in the cells of Lymphoma 6C3HED. Cancer Res 24: 64–69, 1964.

(32) Kakefuda, T., Tanaka, K. K., and Roberts, E.: Electron microscopic studies of mouse ascites tumors, E.L.4 and C1498, in susceptible (C57BL) and resistant (B10.D2) mice. Cancer Res 25: 329–353, 1965.

(33) Dmochowski, L., Grey, C. E., Dreyer, D. A., Sykes, J. A., Langford, P. L., and Taylor, H. G.: Mycoplasma [pleuropneumonia-like organisms (PPLO)] and human leukemia. Med Rec Ann (Houston) 57: 563–568, 1964.

(34) van Iterson, W., and Ruys, A. C.: The fine structure of the Mycoplasmataceae (microorganisms of the pleuropneumonia group PPLO). I. Mycoplasma hominis, M. fermentans and M. salivarium. J Ultrastruct Res 3: 282–301, 1960.

(35) Edwards, G. A., and Fogh, J.: Fine structure of pleuropneumonia-like organisms in pure culture and in infected tissue culture cells. J Bact 79: 267–276, 1960.

(36) Domermuth, C. H., Nielsen, M. H., Freundt, E. A., and Birch-Anderson, A.: Ultrastructure of mycoplasma species. J Bact 88: 727–744, 1964.

(37) Anderson, D. R., and Barile, M.: Ultrastructure of mycoplasma hominis. J Bact 90: 180–192, 1965.

(38) Hummeler, K., Armstrong, D., and Tomassini, N.: Cyptopathogenic mycoplasma associated with two human tumors. II. Morphological aspects. J Bact 90: 511–516, 1965.

(*39*) Hummeler, K., Tomassini, N., and Hayflick, L.: Ultrastructure of a Mycoplasma (Negroni) isolated from human leukemia. J Bact 90: 517–523, 1965.

(*40*) Klieneberger-Nobel, E.: Pleuropneumonia-like organisms (PPLO) Mycoplasmataceae. New York, Academic Press, Inc., 1962.

(*41*) Dmochowski, L., Taylor, H. G., Grey, C. E., Dreyer, D. A., Sykes, J. A., Langford, P. L., Rogers, T., Shullenberger, C. C., and Howe, C. D.: Viruses and mycoplasma (PPLO) in human leukemia. Cancer 18: 1345–1368, 1965.

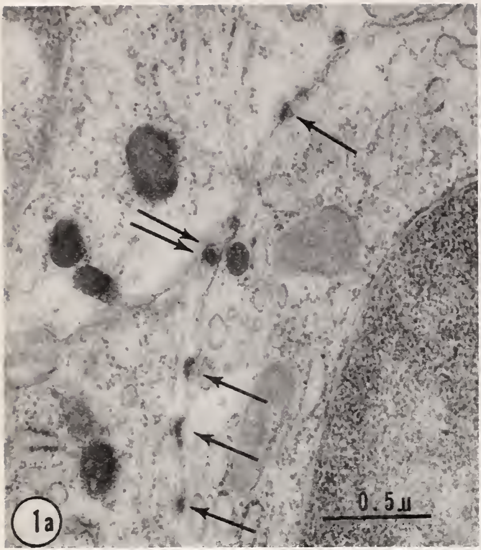
(*42*) Sotelo, J. R., and Porter, K. R.: An electron miscroscope study of the rat ovum. J Biophys Biochem Cytol 5: 327–341, 1959.

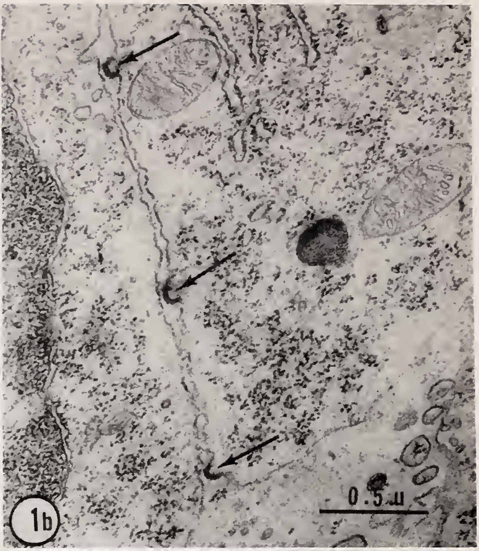
(*43*) Nilsson, O.: Ultrastructure of mouse uterine surface epithelium under different estrogenic influences. 2. Early effect of estrogen administered to spayed animals. J Ultrastruct Res 2: 73–95, 1958.

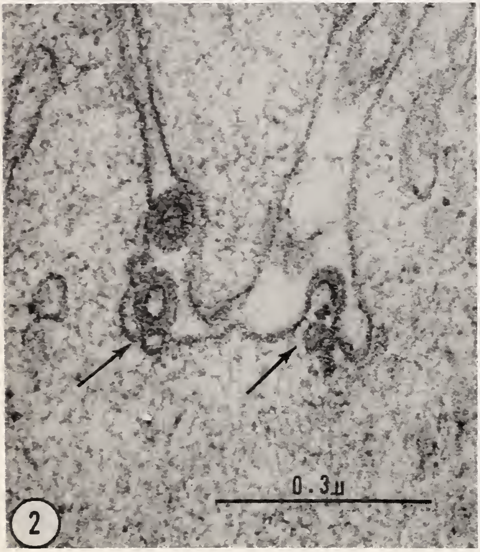
(*44*) Moe, H., Rostgaard, J., and Behnke, O.: On the morphology and origin of virgin lysosomes in the intestinal epithelium of the rat. J Ultrastruct Res 12: 396–403, 1965.

(*45*) Low, F. N., and Freeman, J. A.: Electron Microscopic Atlas of Normal and Leukemic Human Blood. New York, Toronto, and London, McGraw-Hill, 1958.

(*46*) Seman, G., and Dmochowski, L.: Studies on the possible transmission of virus and mycoplasma in leukemia. Med Rec Ann (Houston) 58: 400–405, 1965.







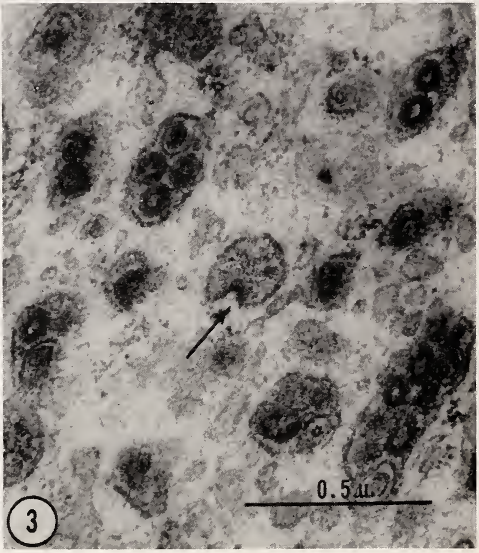
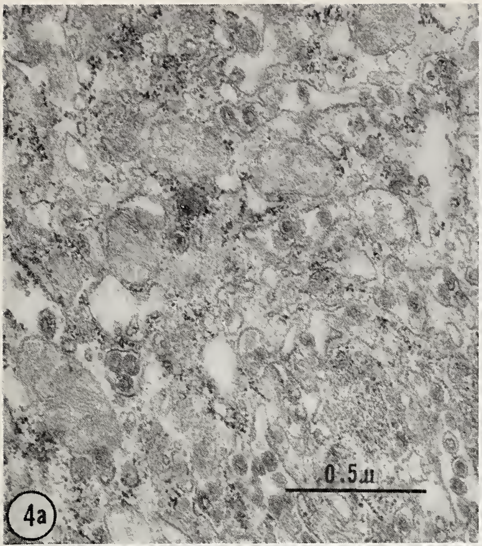


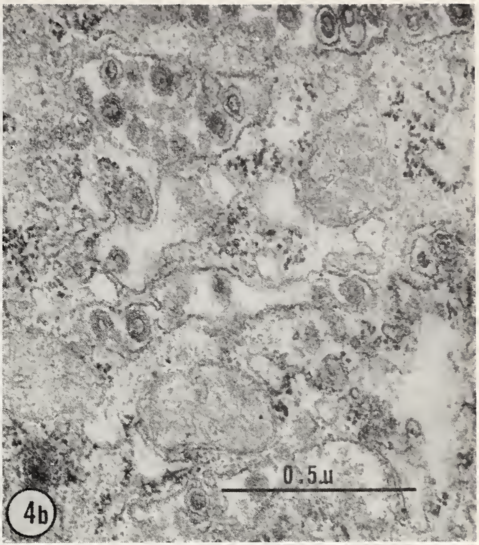
FIGURE 1a.—Part of intercellular space between 3 leukemic cells of the mesenteric lymph node from AKR mouse with lymphatic leukemia after inoculation with Friend virus. Budding from plasma membrane of leukemic cells may be seen (*arrows*). Mature virus particle (*double arrows*). × 30,000

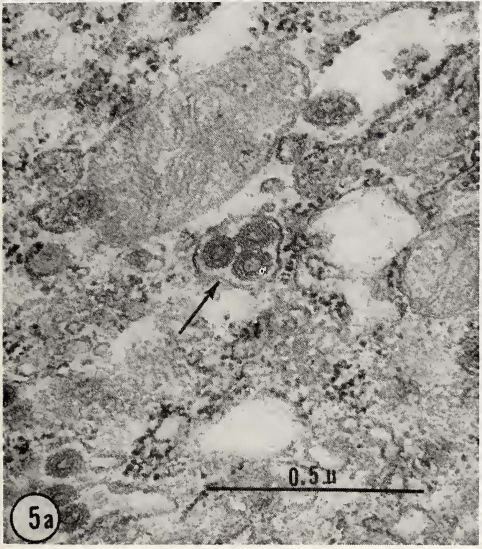
FIGURE 1b.—Part of intercellular space between 2 leukemic cells of the mesenteric lymph node from AKR mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Three buddings of the plasma membrane of one leukemic cell may be seen (*arrows*). × 30,000

FIGURE 2.—Part of cytoplasmic channels of a megakaryocyte of bone marrow from BALB/c mouse with lymphatic leukemia after inoculation with Moloney virus. Two buddings of the membrane of the cytoplasmic channels may be seen (*arrows*). × 90,000

FIGURE 3.—Part of cytoplasm of a megakaryocyte of spleen from BALB/c mouse with lymphatic leukemia after inoculation with Moloney and Passage A (Gross) viruses. Specific granule of a megakaryocyte shows characteristic budding (*arrow*). × 52,500







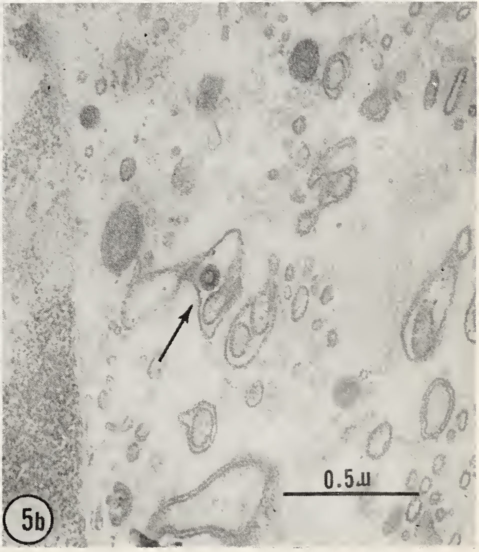
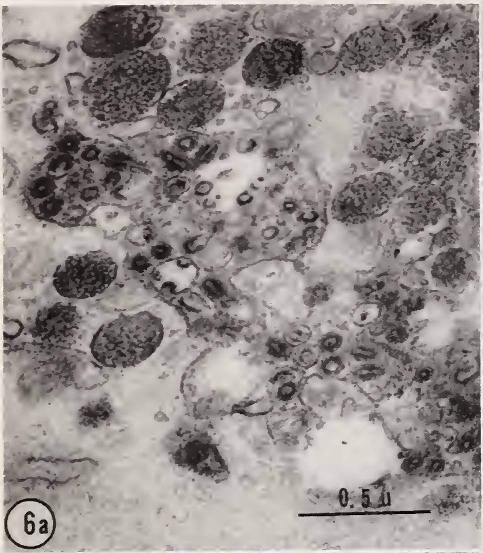


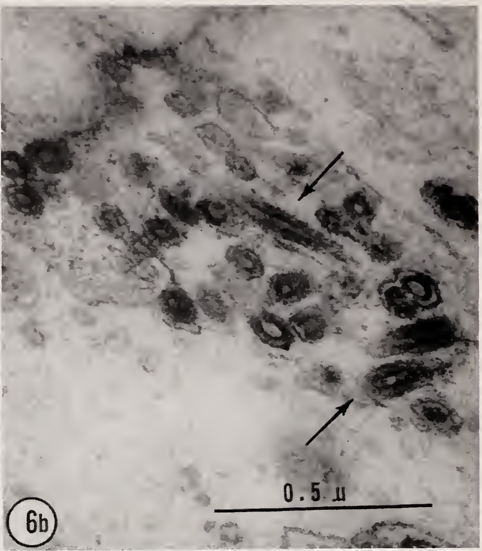
Figure 4a.—Part of the cytoplasm of a megakaryocyte of spleen from BALB/c mouse with lymphatic leukemia after inoculation with Passage A (Gross) and Rauscher viruses. Numerous immature virus particles are present in cytoplasmic channels. × 37,500

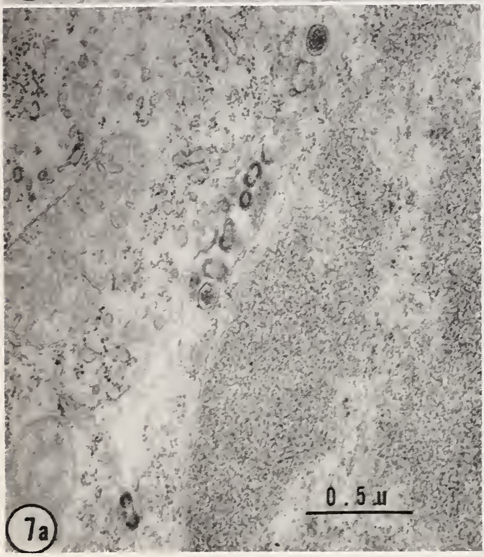
Figure 4b.—Higher magnification of part of the cytoplasm of a megakaryocyte from spleen of BALB/c mouse with lymphatic leukemia after inoculation with Passage A (Gross) and Rauscher viruses. Several immature virus particles having multiple membrane may be seen. × 52,500

Figure 5a.—Part of the cytoplasm of a megakaryocyte from spleen of BALB/c mouse with lymphatic leukemia after inoculation with Passage A (Gross) and Rauscher viruses. Three immature virus particles exhibit continuity of their external membrane forming a short chain (*arrow*). × 60,000

Figure 5b.—Part of the cytoplasm of an epithelial cell from thymus of BALB/c mouse with lymphatic leukemia after inoculation with Moloney virus. Immature virus particle (*arrow*) present in a cytoplasmic vacuole. × 37,500







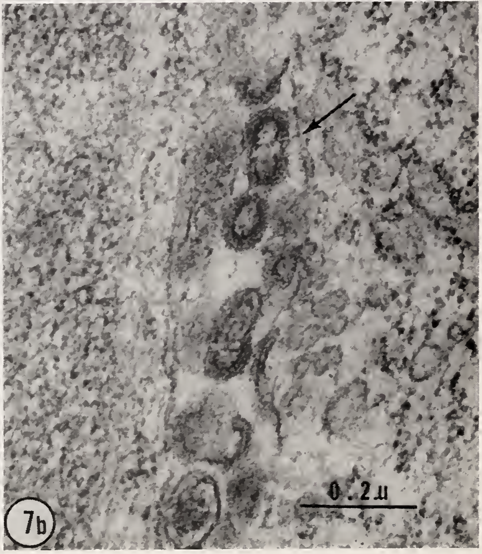


Figure 6a.—Part of the cytoplasm of a megakaryocyte from spleen of BALB/c mouse with lymphatic leukemia after inoculation with Passage A (Gross) and Moloney viruses. In the cytoplasm, specific granules and vacuoles show the presence of cylindrical forms of virus particles. × 37,500

Figure 6b.—Part of the cytoplasm of a megakaryocyte from liver of BALB/c mouse with lymphatic leukemia after inoculation with Moloney virus. Cylindrical forms of virus particles are present in vacuoles (*arrows*). × 60,000

Figure 7a.—Part of the intercellular space between 2 cells from bone marrow of AKR mouse with lymphatic leukemia after inoculation with Moloney virus. Immature and dumbbell forms of virus particles may be seen. × 30,000

Figure 7b.—The same as that shown in figure 7a but at higher magnification. Part of the intercellular space from bone marrow of AKR mouse with lymphatic leukemia. Dumbbell forms may be seen (*arrow*). × 82,500

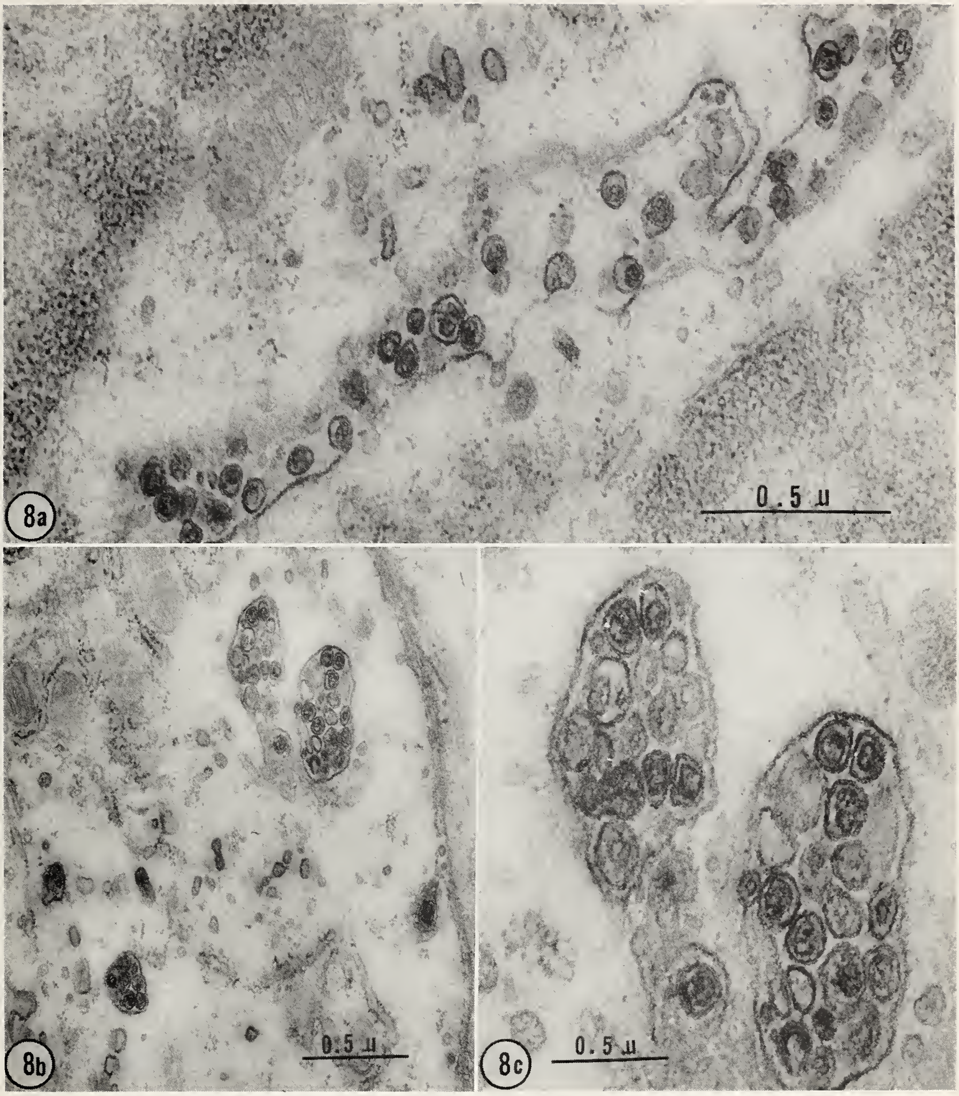
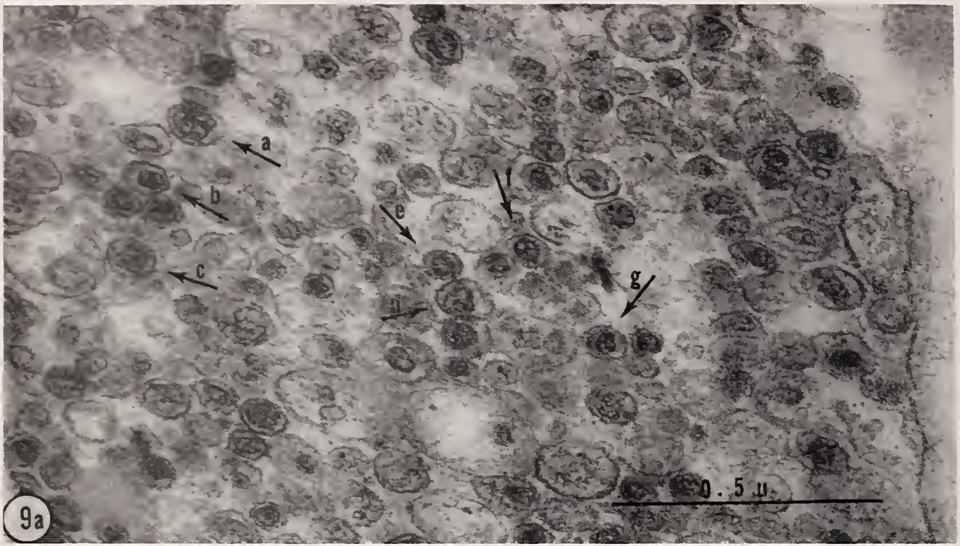


FIGURE 8a.—Part of the intercellular space between 2 leukemic cells from axillary lymph nodes of AKR mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Many mature virus particles may be seen. × 52,500

FIGURE 8b.—Part of the cytoplasm of a reticulum cell from axillary lymph node of AKR mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Three inclusion bodies containing virus particles are present. × 30,000

FIGURE 8c.—Same as that shown in figure 8b but at higher magnification. Inclusion bodies containing virus particles. × 82,500



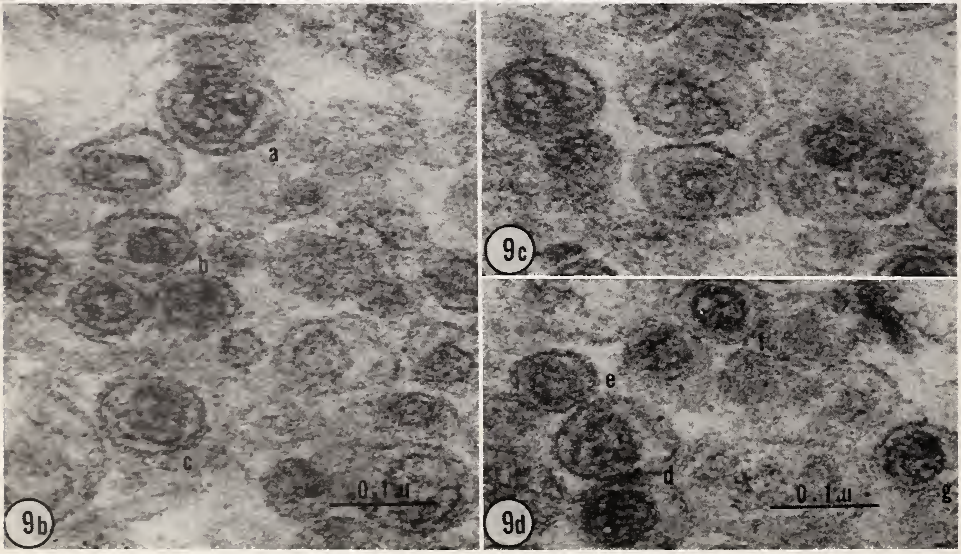
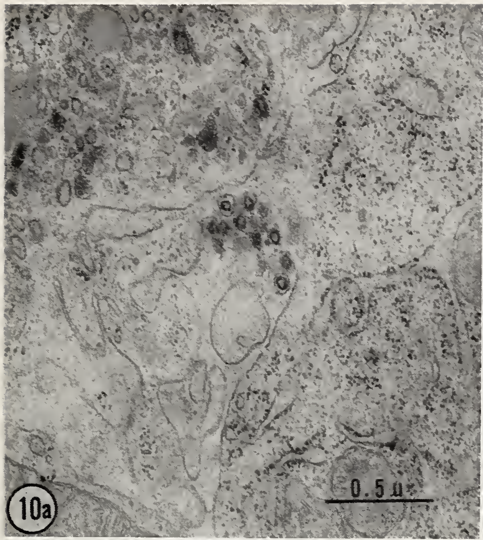
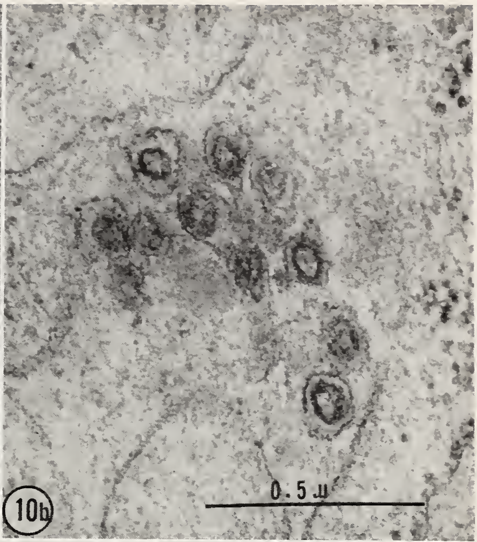
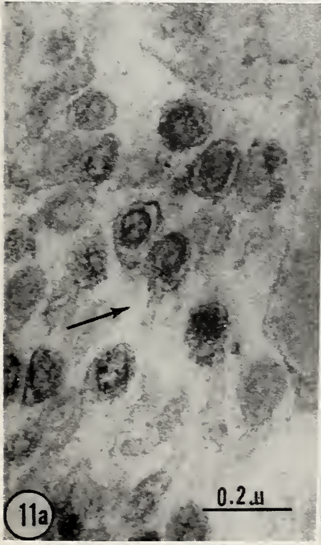


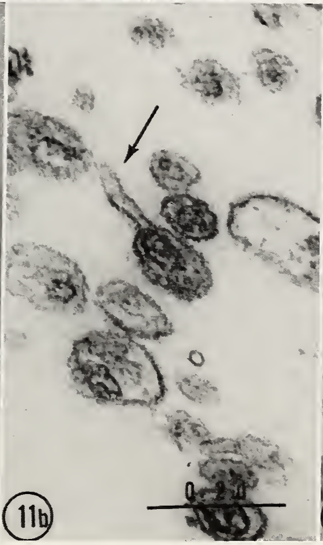
Figure 9a.—Part of intercellular space from spleen of AKR mouse with lymphatic leukemia after inoculation with Moloney virus. Pleomorphism of mature virus particles may be seen (*arrows*). × 52,500

Figures 9b, 9c, and 9d.—Mature virus particles showing various shapes and sizes at higher magnification of figure 9a. × 150,000









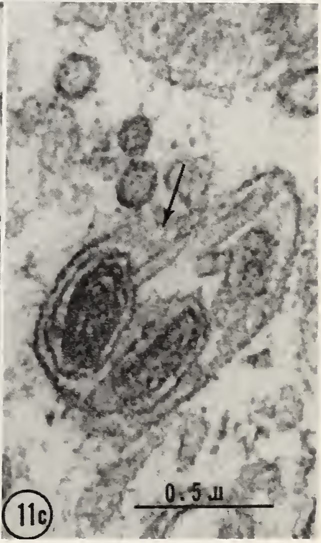


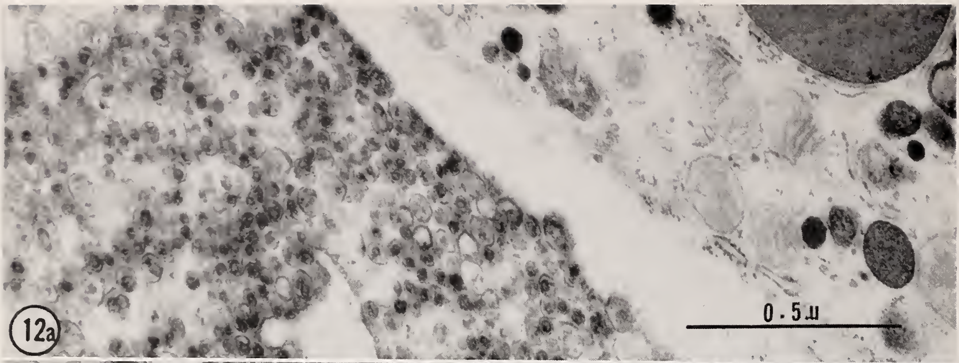
FIGURE 10a.—Part of an intercellular space between a plasma cell and a reticulum cell of liver from AKR mouse with lymphatic leukemia after inoculation of Friend (AKR) virus. Mature virus particles with electron-lucent centers and ring-shaped electron-dense nucleoids may be seen (type C-2 virus particles). × 30,000

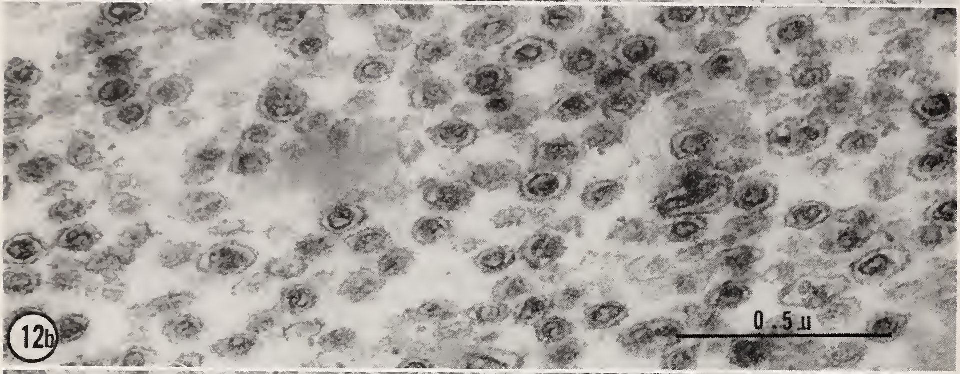
FIGURE 10b.—Type C-2 virus particles. Higher magnification of figure 10a. × 90,000

FIGURE 11a.—Part of a group of mature virus particles in an intercellular space of mesenteric lymph node from AKR mouse with lymphatic leukemia after inoculation of Friend virus from an AKR mouse. Virus particle with tail-like structure may be seen (*arrow*). × 60,000

FIGURE 11b.—Part of a blood-vessel lumen of spleen from AKR mouse with lymphatic leukemia induced by Moloney virus. Virus particle with tail-like structure is present (*arrow*). × 97,500

FIGURE 11c.—Part of the cytoplasm of a reticulum cell of bone marrow from AKR mouse with lymphatic leukemia (Moloney). Virus particle with tail-like structure within vacuole may be seen. × 97,500





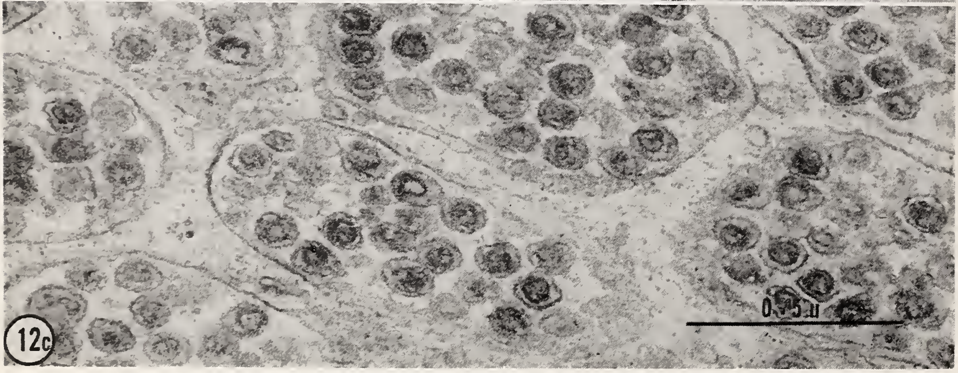
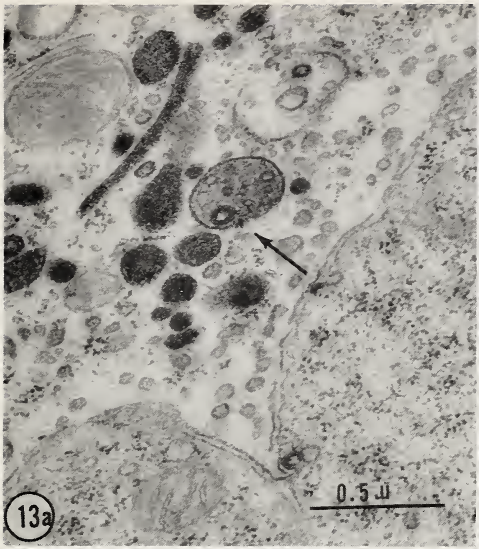
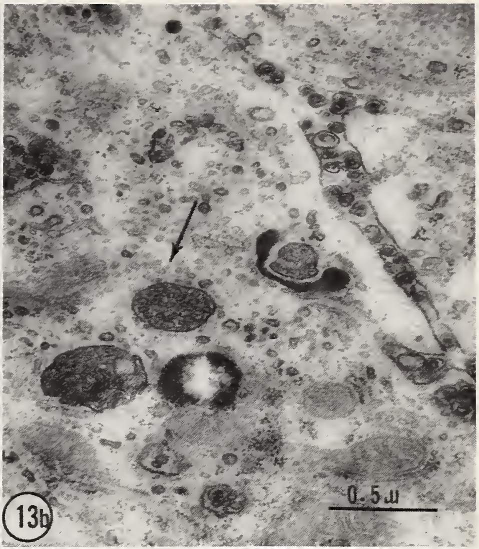


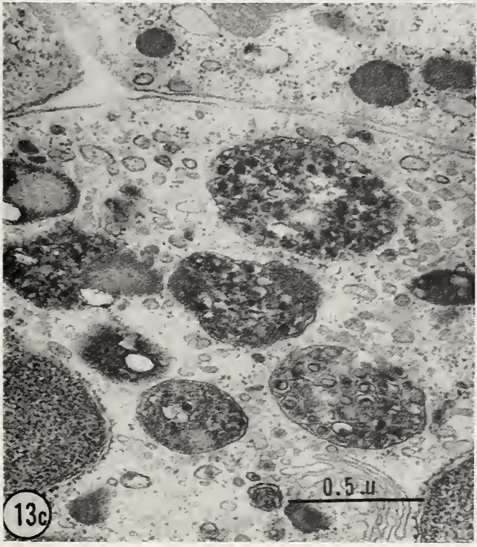
FIGURE 12a.—Part of the intercellular space of spleen from AKR mouse with lymphatic leukemia induced by Moloney virus. Numerous virus particles are present in the intercellular space. To the *right*, reticulum cells with electron-dense inclusion bodies may be seen. × 60,000

FIGURE 12b.—Part of an intercellular space of mesenteric lymph node of BALB/c mouse with lymphatic leukemia after inoculation of Friend virus. Numerous virus particles are present. × 60,000

FIGURE 12c.—Part of the cytoplasm of a reticulum cell in mesenteric lymph node from AKR mouse with lymphatic leukemia after inoculation with Friend virus. Numerous mature virus particles are present within vacuoles. × 60,000







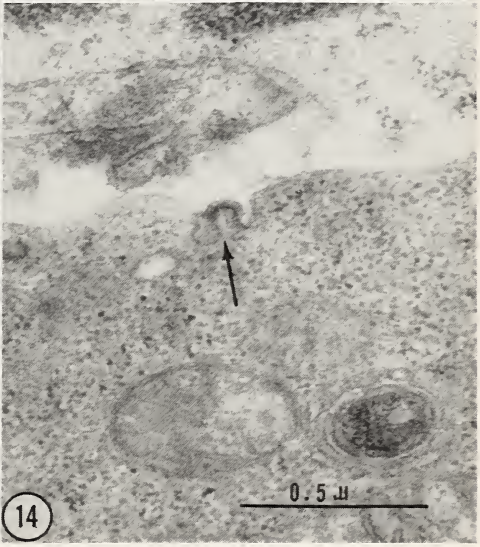
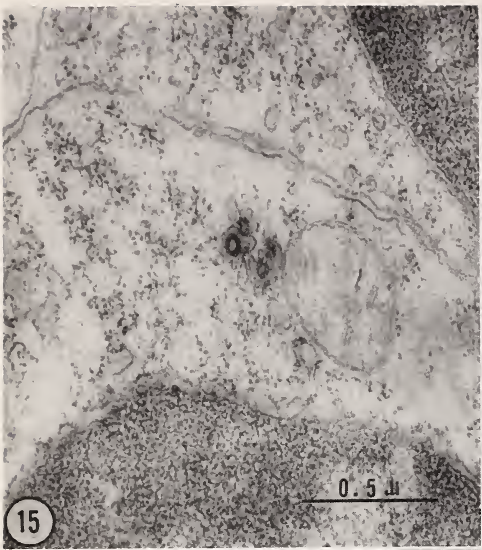


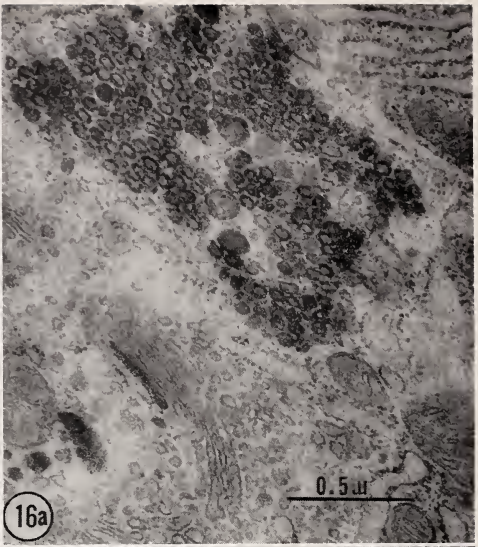
Figure 13a.—Part of the cytoplasm of a reticulum cell of spleen from AKR mouse with lymphatic leukemia after inoculation with Friend virus. Immature virus particles are present in a multivesicular body-like structure and vacuole. Two buddings from the plasma membrane of a cell of the erythroid series may be seen. Many electron-dense inclusions are also present. × 37,500

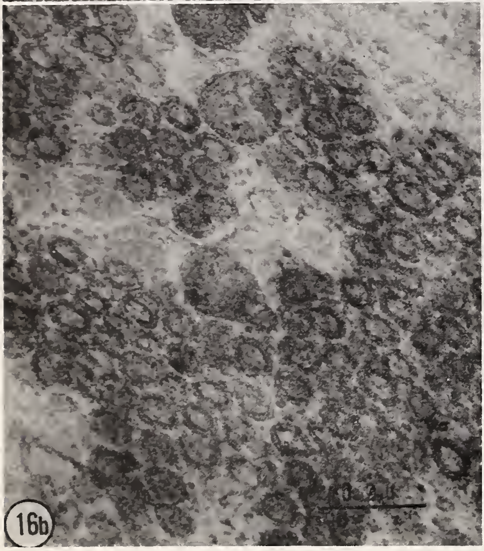
Figure 13b.—Part of the cytoplasm of a reticulum cell of axillary lymph node from AKR mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Multivesicular body-like structure (*arrow*), nonviral inclusion, and a vacuole containing virus particles may be seen. × 30,000

Figure 13c.—Part of the cytoplasm of a reticulum cell of mesenteric lymph node from AKR mouse with lymphatic leukemia after inoculation with Friend virus. Viral and nonviral inclusion bodies are present. × 37,500

Figure 14.—Part of an erythroid cell of spleen from BALB/c mouse with lymphatic leukemia after inoculation with Moloney viruses. Budding from plasma membrane may be seen (*arrow*). × 52,500







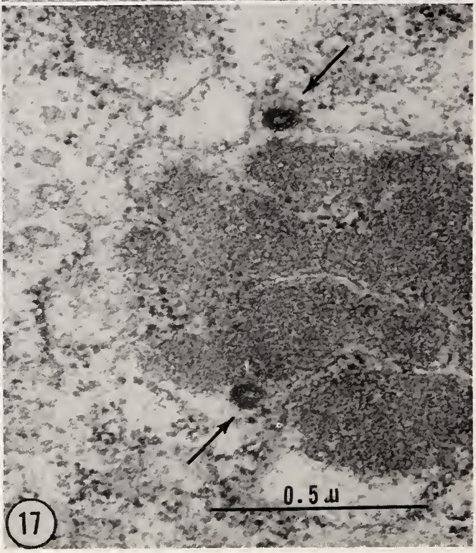
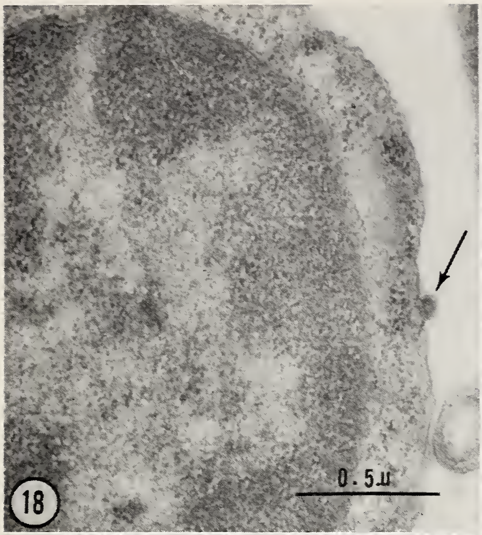


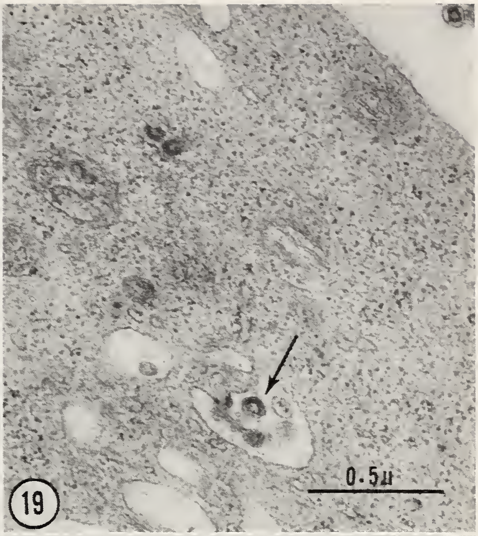
Figure 15.—Part of the cytoplasm of a leukemic cell of mesenteric lymph node from AKR mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Virus particles with 2 concentric membranes may be seen in association with endoplasmic reticulum. × 37,500

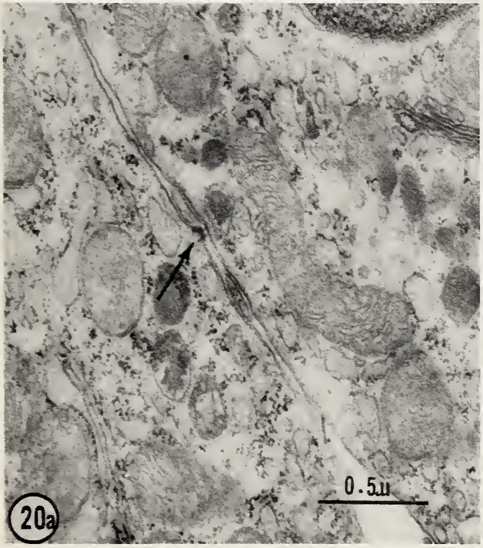
Figure 16a.—Part of the cytoplasm of a plasma cell of mesenteric lymph node from AKR mouse after inoculation with Friend virus. Aggregates of particles with 2 concentric membranes are present. × 34,500

Figure 16b.—Type A virus particles with 2 concentric membranes shown in figure 16a but at higher magnification. The particles are regular in size and the inner membrane is slightly more electron dense than the outer membrane. × 75,000

Figure 17.—Part of the cytoplasm of a plasma cell from BALB/c mouse with lymphatic leukemia after inoculation with Moloney and Passage A (Gross) viruses. Two virus particles with 2 concentric membranes may be seen associated with endoplasmic reticulum (*arrows*). In the endoplasmic reticulum, electron-dense secretory granules are present. × 60,000







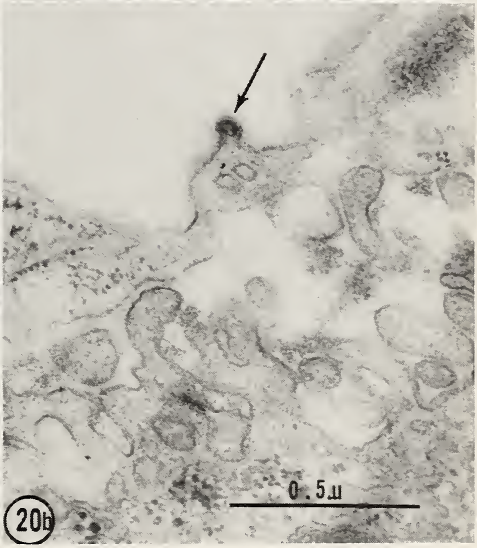
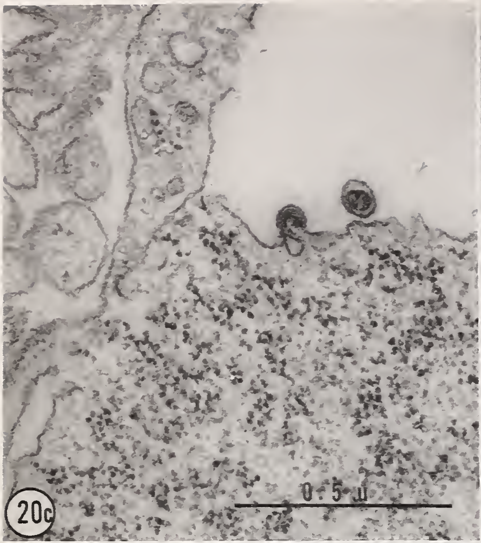


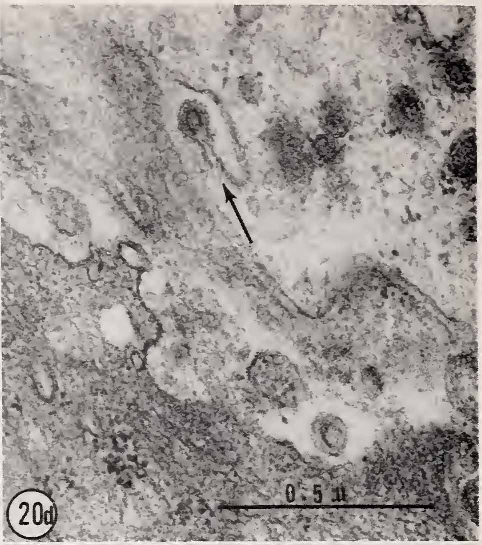
FIGURE 18.—Part of the plasma membrane of an erythroid cell of bone marrow from BALB/c mouse with erythroid leukemia after inoculation with Moloney and Friend viruses. Budding from plasma membrane may be seen (*arrow*). × 40,000

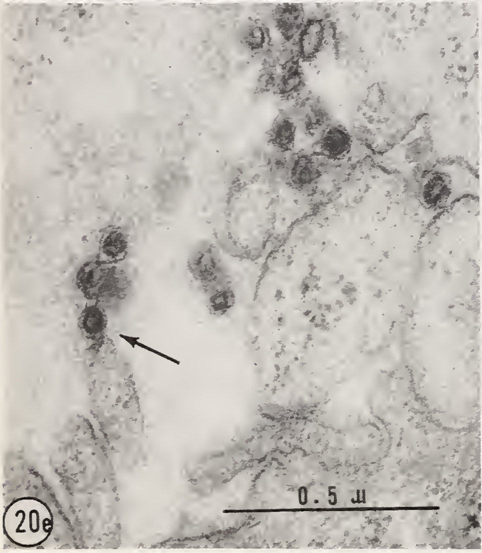
FIGURE 19.—Part of the cytoplasm of an erythroid cell of liver from BALB/c mouse with erythroid leukemia after inoculation with Passage A (Gross) and Friend viruses. Budding from membrane of vacuoles may be seen (*arrow*). × 37,500

FIGURE 20a.—Part of the intercellular space between 2 reticulum cells of bone marrow from BALB/c mouse with erythroid leukemia after inoculation with Moloney and Friend viruses. Budding from plasma membrane of the reticulum cells may be seen (*arrow*). Several electron-dense inclusion bodies are present within cytoplasm of these reticulum cells. × 30,000

FIGURE 20b.—Part of the Disse space of liver from BALB/c mouse with erythroid leukemia after inoculation with Friend and Rauscher viruses. Budding from plasma membrane of endothelial cells into portal vessels may be seen (*arrow*). × 52,500







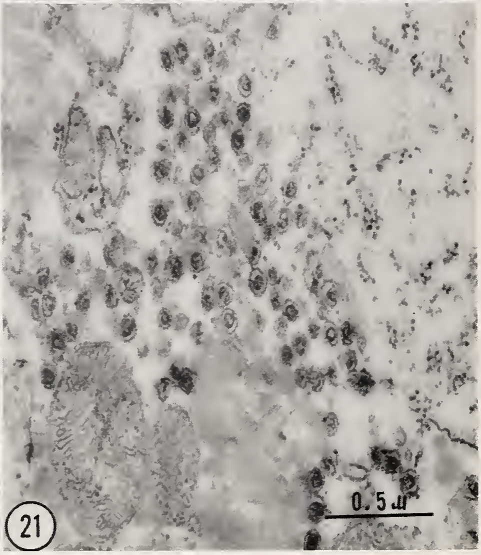
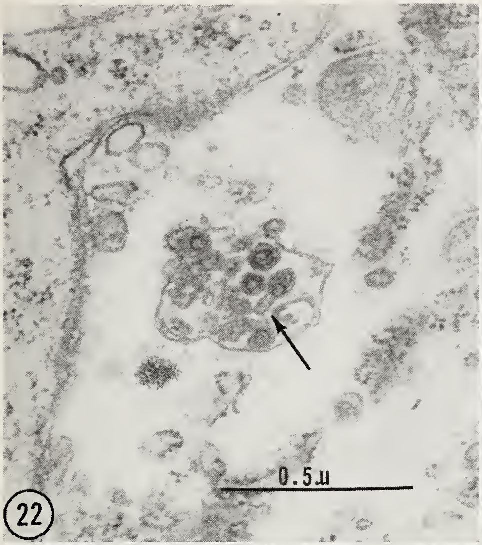


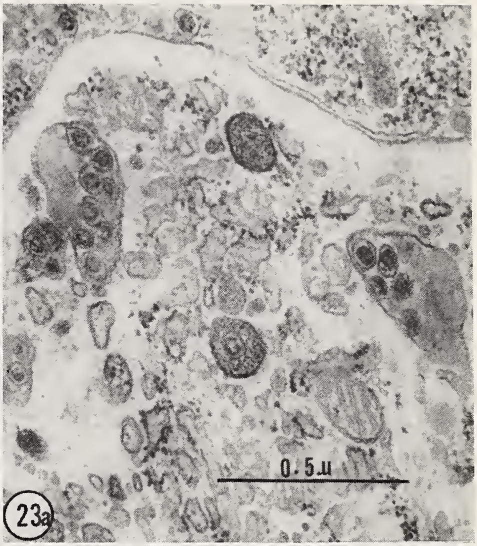
Figure 20c.—Part of the cytoplasm of a Kupffer cell of liver from BALB/c mouse with erythroid leukemia after inoculation with Moloney and Friend viruses. Budding from plasma membrane of the Kupffer cell and mature virus particle within blood vessels may be seen. × 60,000

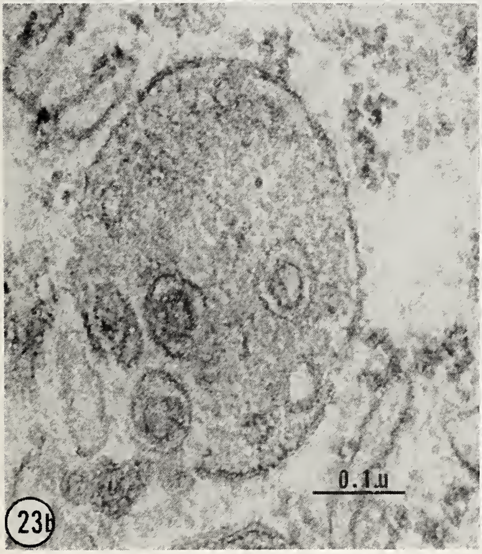
Figure 20d.—Part of the cytoplasm of a Kupffer cell of liver from BALB/c mouse with erythroid leukemia after inoculation with Moloney and Friend viruses. Budding from membrane of vacuole and several electron-dense inclusion bodies are present. × 60,000

Figure 20e.—Part of the Disse space of liver from AKR mouse with erythroid leukemia after inoculation with Friend virus. Budding from plasma membrane of microvilli of the liver cell into Disse space may be seen (*arrow*). 60,000

Figure 21.—Part of the intercellular space of lymph node from BALB/c mouse with erythroid leukemia after inoculation with Friend virus. Mature virus particles of prominent C-2 type are present. × 30,000







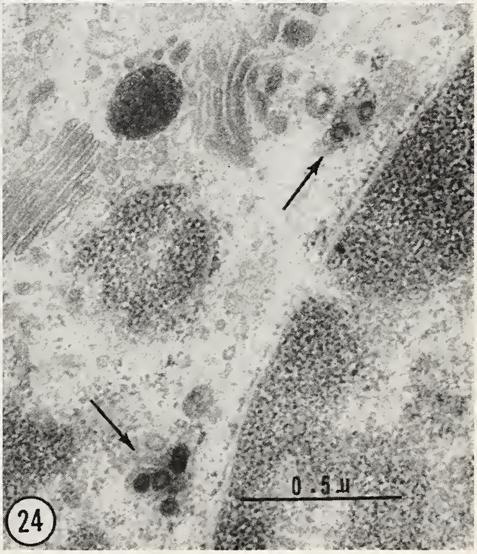


FIGURE 22.—Part of the cytoplasm of a reticulum cell of axillary lymph node from AKR mouse with erythroid leukemia after inoculation with Rauscher virus. Mature virus particles with tail-like structure may be seen in an inclusion body containing virus particles (*arrow*). × 52,500

FIGURE 23a.—Part of the cytoplasm of a reticulum cell of spleen from BALB/c mouse with erythroid leukemia after inoculation with Rauscher virus. Viral and nonviral inclusion bodies are present. × 52,500

FIGURE 23b.—Viral inclusion body (specific granule) in a macrophage of liver from BALB/c mouse with erythroid leukemia after inoculation with Moloney and Friend viruses. Several mature virus particles may be seen. × 135,000

FIGURE 24.—Part of the cytoplasm of a plasma cell of mesenteric lymph node from BALB/c mouse with erythroid leukemia after inoculation with Friend and Moloney viruses. The virus particles with 2 concentric membranes associated with endoplasmic reticulum may be seen (*arrows*). × 45,000

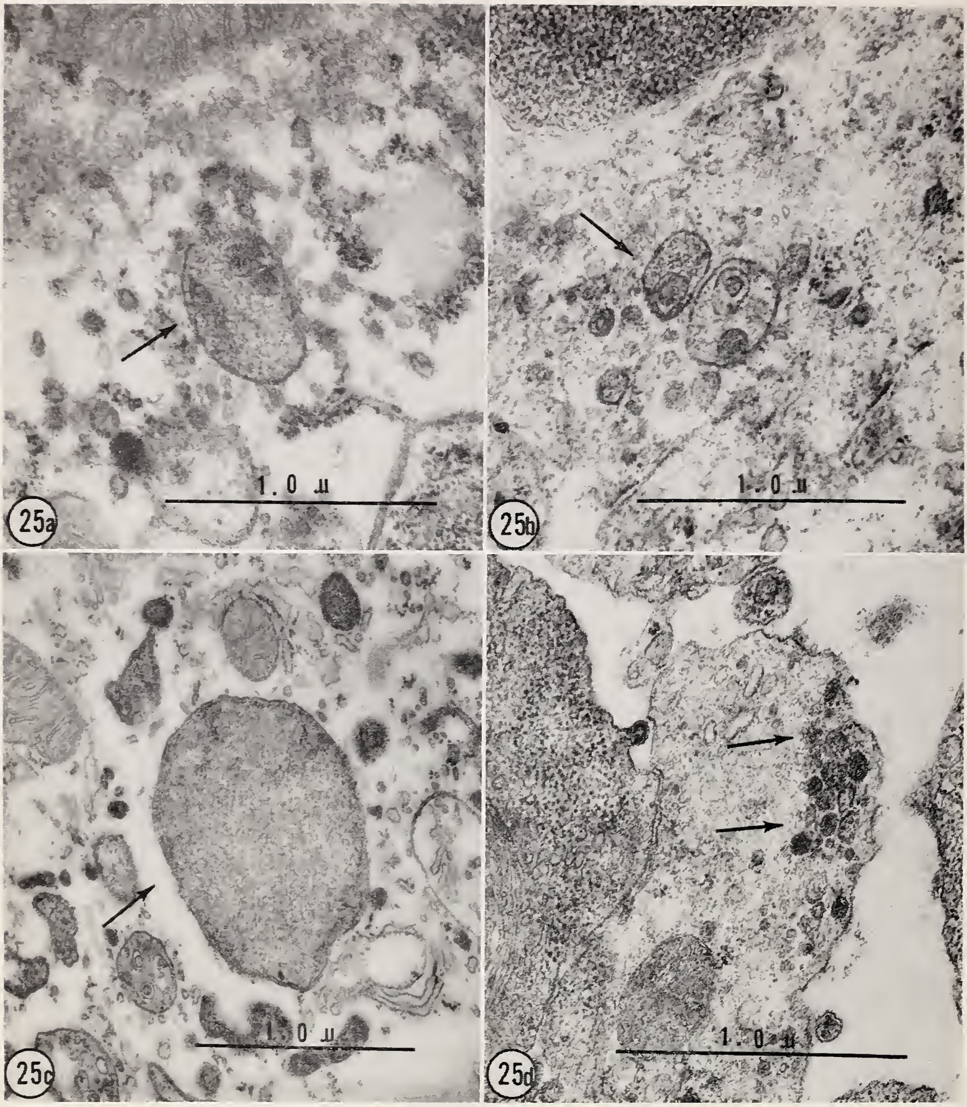
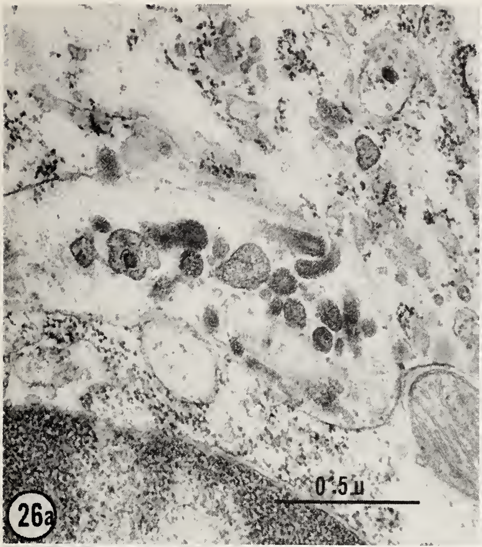


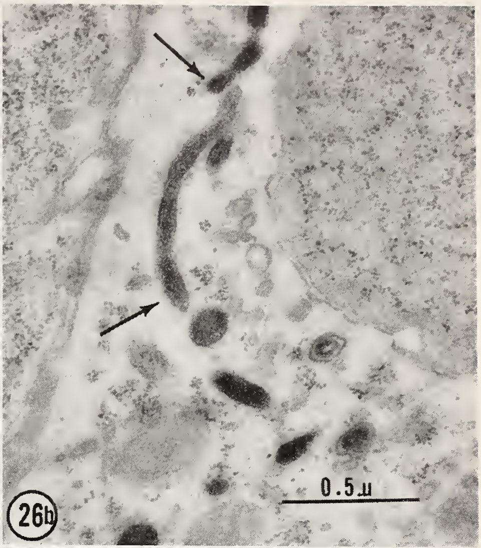
Figure 25a.—Part of the cytoplasm of a macrophage of thymus from C3H/f mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Structure resembling mycoplasma with virus particles may be seen (*arrow*). In this body finely granular protoplasm divided into light and dark zones may be seen. This body may be a cellular constituent. × 37,500

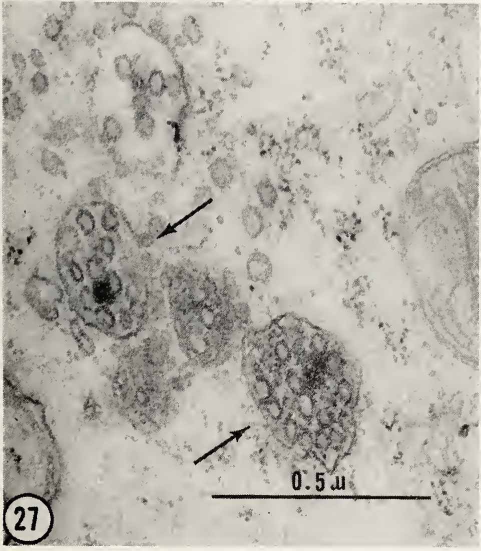
Figure 25b.—Part of the cytoplasm of a macrophage of spleen from BALB/c mouse with lymphatic leukemia after inoculation with Moloney and Passage A (Gross) viruses. Structure resembling mycoplasma containing developmental body is present (*arrow*). × 37,500

Figure 25c.—Part of the cytoplasm of a Kupffer cell of liver from C3H/f mouse with lymphatic leukemia after inoculation with Passage A (Gross) virus. Structure resembling mycoplasma of somewhat irregular shape with fine granules may be seen, but lacking a nuclear area (*arrow*). × 30,000

Figure 25d.—Part of the cytoplasm of a reticulum cell of spleen from BALB/c mouse with erythroid leukemia after inoculation with Friend virus. Structure resembling mycoplasma with small dense bodies may be seen (*arrows*). Budding from the plasma membrane of an erythroid cell may also be seen. × 40,500







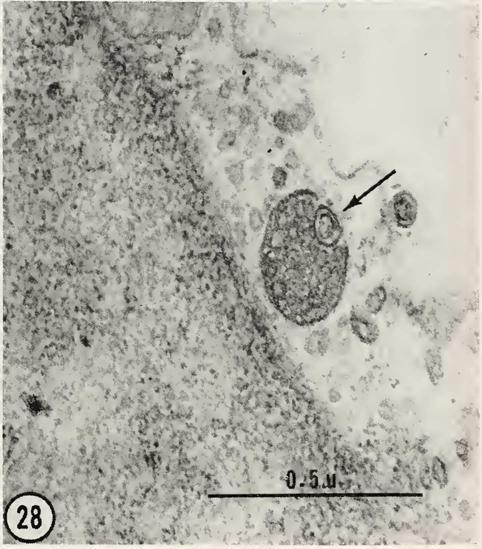


FIGURE 26a.—Part of the cytoplasm of a reticulum cell in spleen from BALB/c mouse with erythroid leukemia after inoculation with Rauscher virus. Structures resembling mycoplasma of various sizes and shapes may be seen. × 40,000

FIGURE 26b.—Part of the cytoplasm of a reticulum cell of spleen from AKR mouse with lymphatic leukemia after inoculation with Friend virus. Structures resembling mycoplasma of electron-dense filamentous shape may be seen (*arrows*). × 37,500

FIGURE 27.—Part of the cytoplasm of a reticulum cell of spleen from BALB/c mouse with erythroid leukemia after inoculation with Friend virus. Large vesicular structures containing many small bodies may be seen (*arrows*). × 60,000

FIGURE 28.—Part of the cytoplasm of a leukemic cell in axillary lymph node from AKR mouse after inoculation with Passage A (Gross) virus. One mature virus particle is present in a large vesicular structure containing many small spherical bodies (*arrow*). × 60,000

DISCUSSION

Dr. Masurovsky: With regard to what has been termed "mycoplasma" in some of the material that Dr. Yumoto presented, might not some of these forms represent organelle-like structures?

Chairman Dmochowski: Indeed that struck us, too, that these structures which we call "resembling mycoplasma" may be cell organelles. There is a recent paper in the *Journal of Ultrastructure* on the epithelial cells of rat intestinum. I was amazed that these people call similar structures, like sausages, virginal lysosomes.

I will not go into the sex life of these organelles or the reasons why they are called so, but I can only say structures like those found in the cytoplasm by Dr. Yumoto have been recently found by us in pure cultures of two mycoplasma: orale and pneumonae. So we can only go by comparison and still are timid even in calling them structures resembling mycoplasma.

A Virus-Induced Rhabdomyosarcoma of Mice[1,2]

John B. Moloney,[3] *Viral Biology Branch, National Cancer Institute,*[4] *Bethesda, Maryland*

THE induction of sarcomas in murine animals by the SV40 virus, the Rous virus, certain of the adenoviruses, and the polyoma agent is well documented. In 1964 Harvey (*1*) described the recovery of a "unidentified virus" from the plasma of rats which had been inoculated with the Moloney strain of leukemia virus. This recovered virus, when inoculated into susceptible mice, rats, and hamsters induced anaplastic sarcomas, angiomas, and leukemias similar to the Rauscher and Friend diseases.

More recently we have described the isolation of a virus that induces rhabdomyosarcomas in mice (*2*). This agent was derived from sarcomas which appeared in BALB/c mice following the inoculation of high doses of mouse-derived Moloney leukemia virus. The agent has been maintained in this strain of mouse for many cell-free tumor generations. The tumor extracts in serial passage were repeatedly (4 times) treated with specific anti-Moloney leukemia virus rabbit serum. After continued "selective virus passage," the agent now induces rhabdomyosarcomas at the site of inoculation in 100% of the recipients within 3 to 5 days after administration. The virus is effective in all strains of mice tested and in selected hybrids of these strains. It is not effective in untreated rats, hamsters, or guinea pigs. However, it can be maintained for extended periods in selected tissue culture systems.

Histologically the virus-induced tumor consisted of elongated cells possessing centrally located and frequently multiple nuclei. The nuclei contained several distinct nucleoli. These characteristic cells resembled myoblasts and some showed distinct cross striations. The cytoplasm of these cells was "tinted" red with Mallory-azan stain. Short, small, fibrosarcomatous-like cells and a few mononuclear round cells were also observed. These characteristics indicate the present classification of the

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This brief presentation was an introduction to the paper by A. J. Dalton entitled, "An Electron Microscopic Study of a Virus-Induced Murine Sarcoma (Moloney)."

[3] With the assistance of E. G. Jenkins and H. Sims.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

tumor as a rhabdomyosarcoma (*3*). No leukemias have been noted in the experimental mice during extended periods of observation.

The virus is stable when stored at the temperature of frozen carbon dioxide and when lyophilized; it is filterable through Selas filters which retain bacteria. It is stable at 37° C for at least 3 hours. However, the virus is inactivated by ether or by heating at 56° C for 30 minutes.

The submicroscopic morphology of the agent is identical to that described for the murine leukemia viruses. Details of the electron microscopic observations on the virus and the induced neoplasm are presented by Dr. A. J. Dalton in an accompanying paper. To further define the physical nature of the virus, Dr. T. O'Connor of the National Cancer Institute has carried out a series of preliminary experiments designed to "band" the agent in potassium citrate gradients. The particles in this medium are concentrated in a fraction which has a density of 1.16 per ml, a density identical to that of the known murine leukemia viruses.

"*In vitro*" neutralization tests were carried out in an effort to show some relationships or distinctions between the present agent and those known to induce sarcomas and leukemia in murine animals. Antisera against the adenoviruses, SV40 virus, Rous sarcoma virus, and Moloney leukemia virus were employed in these studies. No decrease in tumorigenic activity was noted in the presence of the SV40, the adenovirus, and the Rous virus antisera used. One antiserum prepared in rabbits against the Moloney leukemia virus was effective in reducing the biological activity of the sarcoma virus as determined by increased latent periods and increased time to death of the recipient hosts. These findings in addition to those of Rowe (*4*) and Dalton (*5*) indicate the existence of immunological and morphological interrelationships between certain of the murine leukemia agents and the sarcoma virus described herein.

It is suggested, therefore, that there exists in the murine system a complex of viruses similar to that described for the avian tumor virus system. Thus, in the murine tumor complex there have been described antigenically related viruses which induce lymphoid leukemia, osteopetrosis (*6*), erythroblastosis, myeloid leukemia, and now sarcomas. Similar antigenically related viruses have been described in the avian tumor system. It is interesting to speculate that if a virus is shown to be etiologically related to human leukemia that perhaps a similar complex of agents exists in association with certain human tumors.

What the absolute relationship of the present agent and the induced neoplasm is to the known leukemia agents, or more specifically to the virus described by Harvey, must await the outcome of definitive biological and immunological studies.

REFERENCES

(*1*) Harvey, J. J.: An unidentified virus which causes rapid production of tumors in mice. Nature (London) 204: 1104–1105, 1964.

(*2*) MOLONEY, J. B.: The application of studies in murine leukemia to the problems of human neoplasia. Joint Zoological Society/World Health Organization Symposium on Comparative Medicine. London, Academic Press Inc., 1966.

(*3*) PERK, K., and MOLONEY, J. B.: Pathogenesis of a virus-induced rhabdomyosarcoma in mice. J Nat Cancer Inst 37: November, 1966.

(*4*) ROWE, W. P., HARTLEY, J. W., and CAPPS, W. I.: Tissue culture and serologic studies of mouse leukemia viruses. Nat Cancer Inst Monogr 22: 15–19, 1966.

(*5*) DALTON, A. J.: An electron microscopic study of a virus-induced murine sarcoma (Moloney). Nat Cancer Inst Monogr 22: 143–168, 1966.

(*6*) MERWIN, R. M., and REDMON, L. W.: A skeletal lesion produced in mice by sarcoma 37 grown in diffusion chambers. Proc Amer Assoc Cancer Res 5: 44, 1964.

DISCUSSION

Dr. Harvey: I am very grateful to Dr. Moloney for introducing the subject of our sarcoma agent which was isolated at the Cancer Research Department of the London Hospital Medical College in 1964.

The main difference, as far as I can see, between Dr. Moloney's isolate and our own is that ours causes sarcomas not only in mice but also in rats and hamsters after intraperitoneal or subcutaneous injection. Further, the isolate causes not only the very rapid production of sarcomas at many sites but also, in all 8 strains of mice and 3 strains of rats tested, a disease similar in many respects to that induced by the Rauscher and Friend viruses. No procedure so far tested has separated these effects. I would like to have your comments on that, please Dr. Moloney.

Dr. Dourmashkin of the Imperial Cancer Research Fund Laboratories in London has been examining, by means of the electron microscope, tissue from the tumors and enlarged spleens of mice infected with the sarcoma isolate. He finds what he describes as "C-type" particles in large numbers in the megakaryocytes of the spleen but in the tumor specimens so far examined only few particles. This is surprising because cell-free filtrates prepared from tumor tissue very rapidly produced both tumors and splenomegaly when injected back into baby mice and rats.

Dr. John Moloney: We have done everything we can to free our material of the leukemia virus. We have repeatedly passed isolates in the presence of specific anti-Moloney virus serums. As I mentioned, some of it was supplied by Dr. Pinkel and others were produced in primates. So far we have seen no leukemias during our periods of observation, which have been rather short.

The only lesion we have seen is a sarcoma, which may possibly be classified as a rhabdomyosarcoma. We have attempted repeatedly to induce sarcomas in rats and hamsters with our isolates, but without any success. Dr. Shachat in our laboratory has been able to carry the virus and induce tumors in cortisonized and/or thymectomized adult mice and in rats. We haven't had the success that you have had with hamsters.

Dr. Harvey: I would like to make one other short comment. On three separate occasions I have isolated what appears to be the "normal acting" Moloney leukemogenic virus from tissues of mice which, although injected with the sarcoma isolate, did not develop the rapid disease described. They survived with no visible sign of disease for periods of more than 2 months and eventually became leukemic. These leukemias, both on gross and histological examination, were typical of those induced by MLV. Cell-free preparations of tissues from these leukemic mice produced typical generalized lymphatic leukemia when injected back into baby mice. I feel, therefore, that any comment about the C-type particles that Dr. Dourmashkin and other investigators at the M. D. Anderson Hospital have observed in the tumors and enlarged spleens should be prefaced by saying that what appears to be the Moloney leukemogenic virus has been isolated from some animals injected with filtrates of our sarcoma isolate available. Although the particles causing the tumors and splenomegaly may be identical, morphologically, to MLV, I think we should bear that in mind.

Dr. John Moloney: Dr. Harvey, I wonder if in your case it's a question of dose response. Our own material won't dilute out very much further than 10^{-3} of a 1 gram equivalent material. We did get our material from a very high concentration of Moloney virus material. What your original concentration of rat plasma was I don't know. Perhaps in filtration you are diluting out beyond the capacity of the sarcoma agent to induce the tumor and are, therefore, picking up the leukemia virus.

An Electron Microscopic Study of a Virus-Induced Murine Sarcoma (Moloney) [1]

Albert J. Dalton, *Laboratory of Viral Carcinogenesis, National Cancer Institute,*[2] *Bethesda, Maryland*

WITH the obvious exception of mammary tumors, known neoplasms of the mouse induced by cytoplasmic (RNA) viruses have, until very recently, been restricted to tumors of the hematopoietic system. The neoplasms have been lymphoid (*1*), myeloid (*2*), or stem-cell (*3*) leukemias or reticulum-cell tumors (*4*). However, Harvey has just described a solid tumor of the mouse that is transferred by cell-free extract (*5*), and the present report is primarily concerned with the ultrastructure of the cells of a virus-induced sarcoma first reported earlier this year by Moloney (*6*).

MATERIALS AND METHODS

Plasma pellets and samples of subcutaneous and intraperitoneal tumors induced by cell-free extracts in BALB/c mice were fixed in chrome-osmium, dehydrated in ethyl alcohol, and embedded in a mixture of Epon 812 and Araldite (*7*). Sections were cut on an LKB ultratome and stained with uranyl acetate (*8*) and lead citrate (*9*). Electron micrographs were taken with a Siemens Elmiskop I with a 50 μ objective aperture and 80 kv accelerating voltage.

RESULTS

Plasma pellets were prepared from the blood of mice that had developed localized tumors following either subcutaneous or intraperitoneal inoculation of cell-free extracts of previous generation tumors. This was done because it was known that large amounts of virus are in the blood of mice with virus-induced murine leukemia (*10*), and this particular tumor first appeared in mice inoculated with the Moloney agent (*6*). These pellets, when examined with the electron microscope, contained very few virus particles. Those particles present, intermingled with a great deal of cell debris, were indistinguishable from murine leukemia virus particles. Some particles possessed the outer coat, intermediate layer, and relatively electron-lucent nucleoid characteristic of immature murine leukemia particles. Others possessed the electron-dense nucleoids and tails characteristic of murine leukemia particles when isotonic citrate is used in the isolation procedure (fig. 1).

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

In the first material examined from a subcutaneous tumor, a different type particle was found. This particle appeared in the cytoplasm of a cell containing numerous vesicles bounded by double membranes. The vesicles varied somewhat in size but averaged approximately 0.2 μ in diameter (fig. 2). The particles were present within cisternae lined by a single smooth membrane probably derived from the Golgi complex. They averaged 50 mμ in diameter and contained a nucleoid with a diameter of approximately 30 mμ. The nucleoid itself consisted of an outer electron-dense sphere surrounding a relatively electron-lucent central area. The outer coat of the particles had about the same degree of electron density as that in the inner area of the nucleoid (fig. 3). Other smaller particles with the same size and appearance of the nucleoids of the larger particles were observed in the cytoplasmic matrix between the flattened sacs of the Golgi complex (fig. 4). Occasionally these particles appeared to be budding into the cisternae of the Golgi complex (*arrow* in fig. 4). They were found only in this type of cell in this first specimen and have not been observed in any of the sarcoma material since.

Particles indistinguishable from those of murine leukemia have been observed consistently in the interstices between sarcoma cells (fig. 5). They appeared as buds at the plasma membrane or as mature particles being absorbed by the same cell (fig. 6, A and B). Occasionally buds formed at the surface of vacuoles within the cytoplasm (fig. 7), but more often they formed at the free cell surface (fig. 8). Recently released and still immature particles may be observed in proximity to the cell surface (fig. 9). The particles averaged 100 mμ in diameter, the nucleoids 50 mμ in immature particles. In mice with virus-induced sarcoma, which showed no clinical signs of leukemia, no virus particles were found in lymph node, thymus, or megakaryocytes in the spleen and bone marrow.

The tumor cells themselves appeared to be of two types. Most of them were rather small and spindle shaped (17–20μ long and 4–5μ wide) and contained an elongated nucleus (fig. 10). The ergastoplasm of these cells was well developed, the cisternae of the endoplasmic reticulum tending to be elongated along the long axis of the cell. The cisternae contained a flocculent, moderately electron-dense precipitate. Mitochondrial profiles were usually circular or oval with an average diameter of 0.4μ. Occasional elongated profiles were also observed (fig. 11). The Golgi complex was moderately well developed, possessing the usual three components, vacuoles, small vesicles, and flattened sacs (fig. 12). Interspersed between components of the ergastoplasm and mostly oriented along the long axis of the cell were groups of fibrils approximately 70 A in diameter. Occasionally longitudinal bundles of fibrils associated with a finely granular matrix were found either at the cell border (fig. 13) or in a more central location (fig. 14).

The other cell type was basically similar to the first but was much larger, averaging 40μ long and 10μ wide, containing one or more nuclei with circular profiles (fig. 15). The ergastoplasm in these cells was also well developed, but the cisternae of the endoplasmic reticulum did not show a longitudinal orientation. The cisternae did, however, contain a moderately electron-dense, flocculent precipitate (fig. 16). The mitochondria were more variable in size than those of the first cell type, having fewer cristae, and their matrix was relatively electron lucent. The Golgi complex was less well developed and occupied a smaller percentage of the cytoplasmic area than it did in the first cell type (fig. 17). The cytoplasmic matrix of these cells appeared to be more electron lucent than that of the first cell type and contained fewer free ribosomes (figs. 16 and 17). Numerous fibrils were in the cytoplasmic matrix of these cells and in some cases were very prominent (fig. 17). In the material examined there were no clear-cut examples of cell types transitional between the two just described.

Associated with subcutaneous tumor cells were isolated skeletal muscle cells containing typical nuclei, mitochondria, and Golgi complexes (fig. 18). A thin border of moderately electron-dense, granular material similar to basement membrane substance appeared to be attached to the sarcolemma of the muscle cells (fig. 19). This granular material was not associated with the plasma membranes of tumor cells. Mitochondria within the muscle fibers showed the usual orientation in relation to the myofibrils, and elements of the sarcoplasmic reticulum were evident (fig. 19); however, the regular orientation of the myofibrils was disturbed in these isolated muscle cells. "Z" lines were obvious as were the myofilaments, but A and I bands were indistinct (fig. 20). No evidence has been found of innervation of these isolated muscle cells. Electron-dense particles were frequently present within cisternae of the sarcoplasmic reticulum (fig. 21). While most of these particles were type A-1 (*11*) (fig. 21), some were typical C type particles, some budding from the sarcolemma tubules (T system) (fig. 22), others free and immature (fig. 23), and still others, mature particles with electron-dense centers (fig. 24), lying free in the cisternae.

DISCUSSION

Failure to find the expected amount of virus in plasma pellets appears to correlate with the fact that, in mice with virus-induced sarcoma but free of clinical signs of leukemia, no virus particles were detected in thin sections of the thymus, lymph node, and megakaryocytes of the spleen. It might be expected that, in mice with both sarcoma and leukemia, virus would be found in both the plasma and hematopoietic organs. To date, however, we have not examined such material.

The virus particles observed in plasma pellets, while small in number, were indistinguishable from the particles in plasma pellets of mice and rats with virus-induced leukemia. The particles averaged 100μ in diameter. Some identified as immature particles possessed an intermediate membrane and a nucleoid averaging 50μ in diameter, the center of which was electron lucent. Others identified as mature particles possessed straight, stout tails when the original whole blood was diluted with isotonic (5% or 0.153 M) citrate. Recent evidence (*12*) has been interpreted as indicating that these tails are artifacts induced by exposure to a hypertonic environment. More recently it has been shown that tail formation is a reversible phenomenon (*13*). There is no question but what the outer coat of the mature murine leukemia virus particle functions as a very sensitive osmometer. It is also clear that tails may be induced to form when mature, unfixed particles are dried down in phosphotungstate. Tails induced to form under these conditions may be considered artifacts. However, the fact that tails have been observed on particles fixed *in situ* with hypertonic (*14*), isotonic (*15*), and hypotonic (*16*) fixatives needs to be explained. Also the evidence of Bartl (*17*) that the presence or absence of ATP has a direct bearing on the absence or presence of tails on avian myeloblastosis virus particles (a virus basically similar in morphology to the murine leukemia virus particle) is of interest. Thus the present status of this relatively minor discourse concerning the significance of the tails on murine leukemia virus particles would appear to be that, while tails may be induced to form by experimental intervention (immersion in a hypertonic medium), they may also occur on particles *in situ* with no such treatment and are not necessarily artifacts. It is at least conceivable that tissue fluids, under certain conditions, could be hypertonic to the virus particle outer membrane and could thus induce tail formation.

The first particles found on examination of sections of the subcutaneous sarcoma are unusual both in their location and mode of formation. Apparently small (30 μ) electron-dense bodies develop in the cytoplasmic matrix in the Golgi zone of the cell. They then bud into the Golgi sacs and vacuoles, acquiring a Golgi membrane as an outer coat as they do so. That these particles have been found only in two cells of one sample of tumor and not in any of the many samples of tumor studied since would suggest that they are not related etiologically to the sarcoma. Their significance is thus at the present completely unknown, although their size and morphology are similar to those of the LDH agent (*18*).

Larger mature and immature particles found between sarcoma cells and particles budding from these cells are indistinguishable morphologically from typical murine leukemia virus particles (*19*). That they are produced by the malignant cells and that there is no other likely candidate suggest they are etiologically related to the sarcoma. Our inability to find particles in lymph node, thymus, and megakaryocytes of the spleen and bone would explain, at least in part, the low level of virus in the plasma of mice with virus-induced sarcoma, since it is believed that the high level of virus in the plasma of leukemic mice and rats is partly due to the release of large amounts of virus into the bloodstream at the time of platelet delamination from megakaryocytes.

The presence of two distinct sizes of tumor cells is of interest. While both types contain fibrils, the smaller frequently has bundles of fibrils, whereas the larger exhibits a more even distribution of them. Periodicity in the fiber bundles supports the original pathologic classification of rhabdomyosarcoma (*6*).

The individual isolated skeletal muscle cells, isolated presumably by invasion of sarcoma cells, do not show the precise organization and orientation of functional skeletal muscle fibers. This observation may be related to the failure to find any evidence of innervation of these individual cells. Typical type C particles present within the sarcolemma tubules (T system) is reminiscent of the situation in mice with leukemia in which many non-neoplastic cells of the hematopoietic series as well as plasma cells have been shown to replicate virus (*15, 20, 21*).

SUMMARY

Virus-induced sarcoma and, in addition, plasma pellets and tissues of BALB/c mice carrying the tumor have been studied with the electron microscope. In the first tumor material examined, small (50 μ) particles with the morphology of viruses were observed in various stages of formation in the Golgi zone of a few cells. These particles were not seen in any material studied later. Larger particles indistinguishable from murine leukemia virus particles were observed in small numbers in plasma pellets, as well as budding from sarcoma cells and lying in the interstices between them. These are the particles considered to be etiologically related to the sarcoma. The tumor cells themselves were of two types, one small and spindle-shaped with single elongated nuclei; the other larger with one or more nuclei with circular profiles. Both cell types contained large amounts of ergastoplasm and numerous fibrils. Single, isolated skeletal muscle cells associated with sarcoma growing in a subcutaneous position were observed to contain type C particles, some budding from the sarcolemma tubules (T system)—others lying free in the cisternae.

REFERENCES

(*1*) Gross, L.: "Spontaneous" leukemia developing in C3H mice following inoculation, in infancy, with AK-leukemic extracts, or AK-embryos. Proc Soc Exp Biol Med 76: 27–32, 1951.

(*2*) Graffi, A.: Chloroleukemia of mice. Ann NY Acad Sci 68: 540–558, 1957.

(*3*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307–318, 1957.

(*4*) Stansly, P. G., and Soule, H. D.: Transplantation and cell-free transmission of a reticulum-cell sarcoma in BALB/c mice. J Nat Cancer Inst 29: 1083–1105, 1962.

(*5*) Harvey, J. J.: An unidentified virus which causes the rapid production of tumours in mice. Nature (London) 204: 1104–1105, 1964.

(*6*) Moloney, J. B.: The application of studies in murine leukemia to problems in human leukemia. In Proc Joint Symposium on Some Recent Developments in Comparative Medicine. London, Academic Press Inc. In press.

(*7*) Mollenhauer, H. H.: Plastic embedding mixtures for use in electron microscopy. Stain Tech 39: 111–114, 1964.

(*8*) Gibbons, I. R., and Bradfield, J. R. G.: Experiments on staining thin sections for electron microsocpy. *In* Proceedings Stockholm Conference on Electron Microsocpy. New York, Academic Press Inc., 1957, pp 121–124.

(*9*) Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212, 1963.

(*10*) Dalton, A. J., and Moloney, J. B.: Recovery of virus from the blood of rats with induced leukemia. *In* Symposia Int Soc Cell Biol I. Interpretation of Ultrastructure. New York, Academic Press Inc., 1962, pp 385–392.

(*11*) Dalton, A. J.: Micromorphology of murine tumor viruses and of affected cells. Fed Proc 21: 936–941, 1962.

(*12*) de Harven, E., and Friend, C.: Structure of virus particles partially purified from the blood of leukemic mice. Virology 23: 119–124, 1964.

(*13*) Levy, J.-P., Boiron, M., Silvestre, D., and Bernard, J.: The ultrastructure of the Rauscher virus. Virology 26: 146–150, 1965.

(*14*) Dalton, A. J., Haguenau, F., and Moloney, J. B.: Further electron microscopic studies on the morphology of the Moloney agent. J Nat Cancer Inst 33: 255–275, 1964.

(*15*) Feldman, D. G., and Gross, L.: Electron-microscopic study of the mouse leukemia virus (Gross), and of tissues from mice with virus-induced leukemia. Cancer Res 24: 1760–1783, 1964.

(*16*) Okano, H., Kunii, A., and Furth, J.: An electron microscope study of leukemia induced in rats with Gross virus. Cancer Res 23: 1169–1175, 1963.

(*17*) Bartl, P., Riman, J., and Hart, J.: Some morphological properties of BAI strain A virus particles. *In* Proc Third Europian Regional Conf on Electron Microscopy. Prague, Publishing House of Czech Acad Sci, vol B, 1964, pp 373–374.

(*18*) de-Thé, G., and Notkins, A. L.: Ultrastructure of the lactic dehydrogenase virus (LDV) and cell-virus relationships. Virology 26: 512–516, 1965.

(*19*) Dalton, A. J., Law, L. W., Moloney, J. B., and Manaker, R. A.: An electron microscopic study of a series of murine lymphoid neoplasms. J Nat Cancer Inst 27: 747–791, 1961.

(*20*) Dmochowski, L., Padgett, F., and Gross, L.: An electron microscopic study of leukemia induced with mouse leukemia virus (Gross). Cancer Res 24: 869–899, 1964.

(*21*) Dalton, A. J., Moloney, J. B., Porter, G. H., Frei, E., and Mitchell, E.: Studies on murine and human leukemia. Trans Assoc Amer Physicians 77: 52–63, 1964.

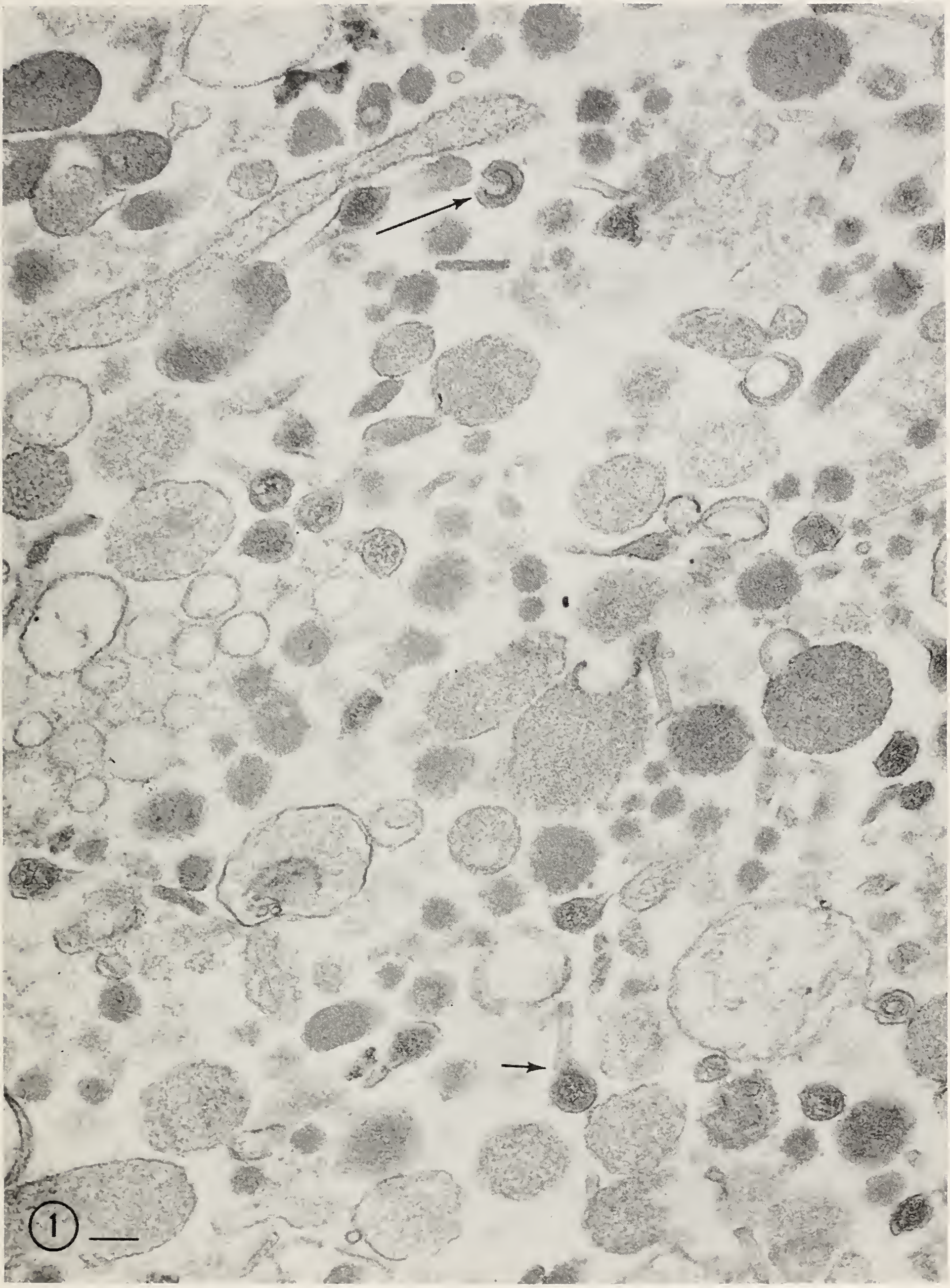


FIGURE 1.—Thin section of a plasma pellet from a mouse with virus-induced sarcoma. Isotonic citrate was used in the isolation procedure. Intermingled with a large amount of cellular debris are mature particles with electron-dense nucleoids and tails (*short arrow*) and one immature particle with an intermediate membrane (*long arrow*). *Horizontal line* represents 0.1 μ. Approximately × 70,000

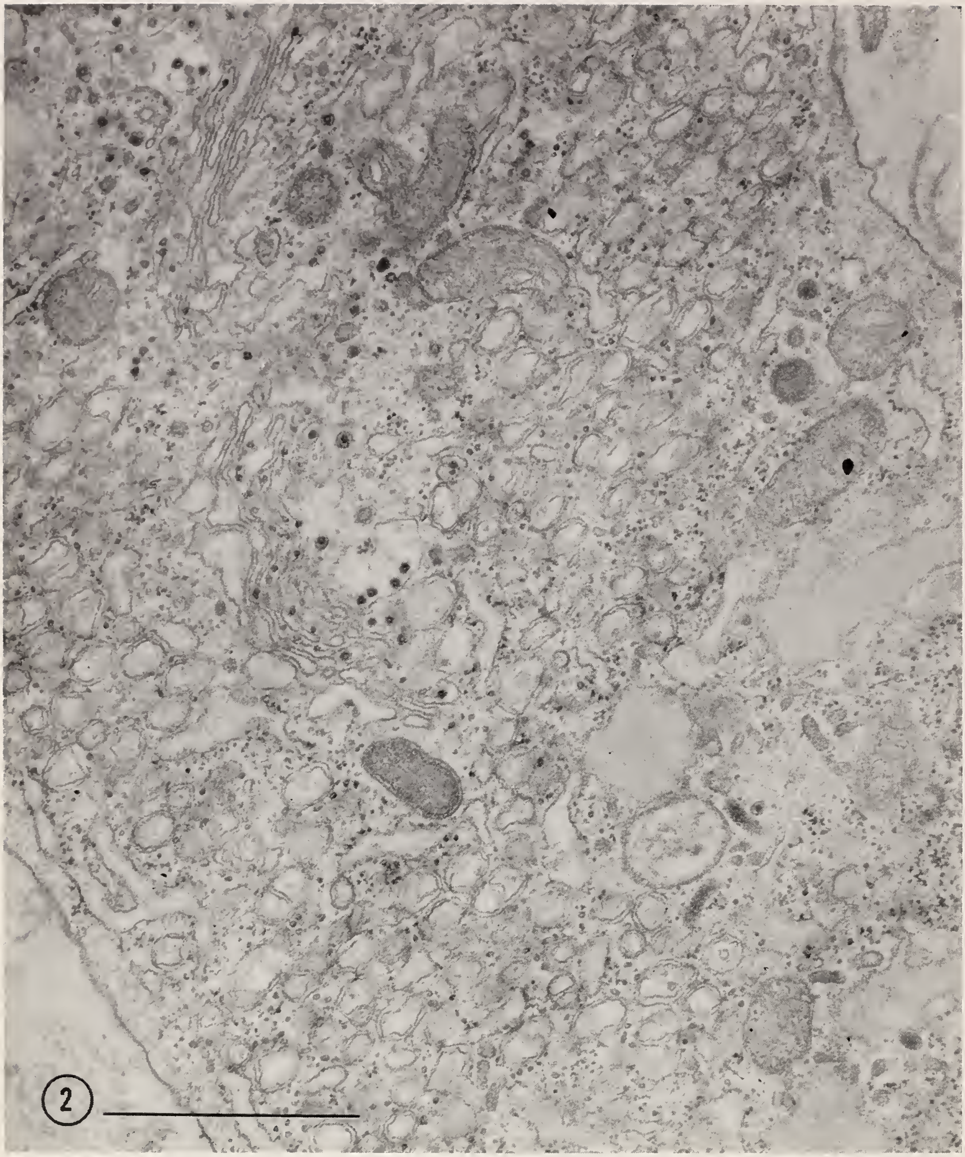


FIGURE 2.—Part of an unusual type of cell containing numerous double-walled vesicles. The Golgi complex containing many small granules is evident. *Horizontal line represents* 1.0 μ. Approximately × 35,000

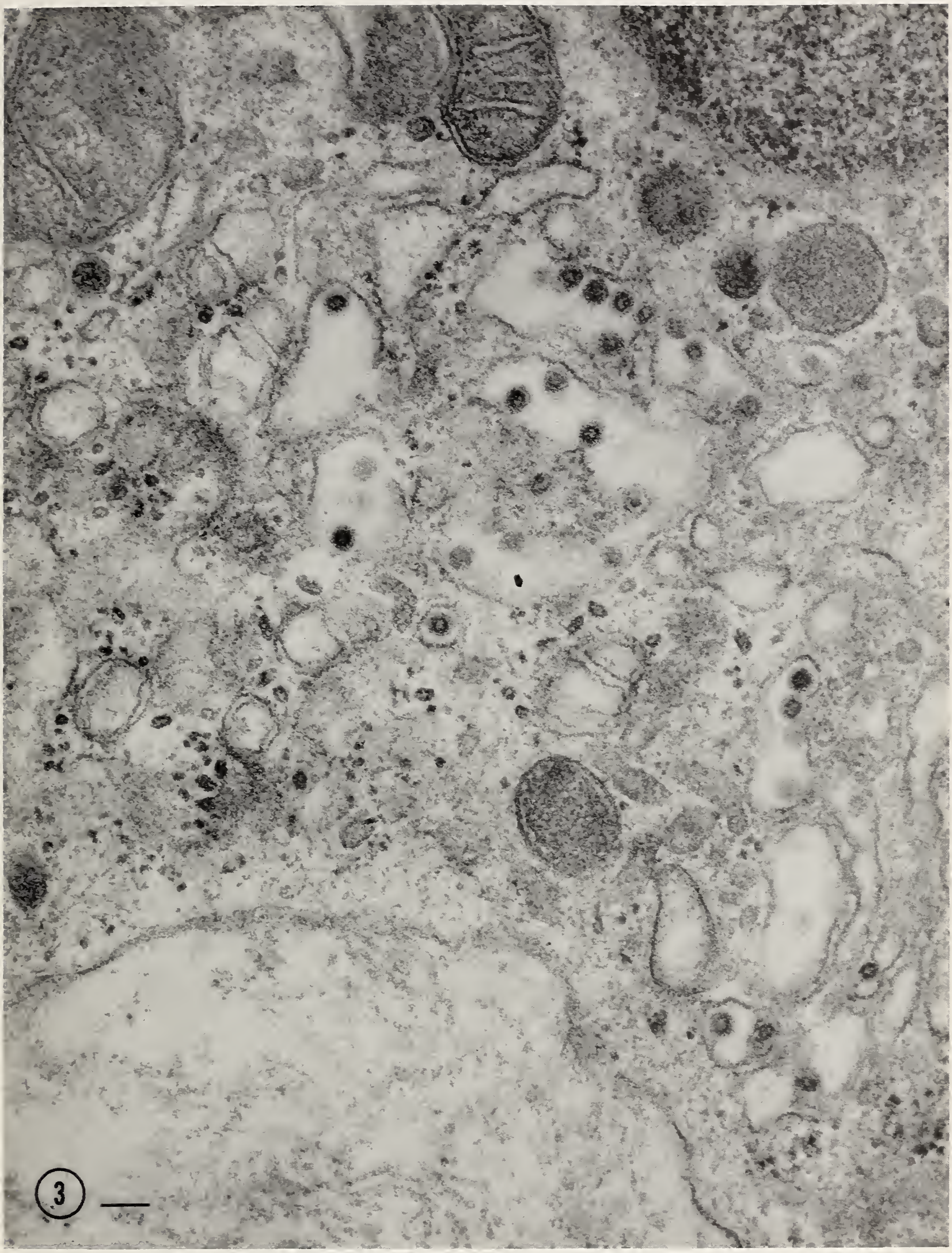


FIGURE 3.—Higher power view of an area of the Golgi complex of a cell similar to that in figure 2. Particles approximately 50 mμ in diameter are present within vacuoles. Their margins are indistinct, but they contain definite electron-dense nucleoids approximately 30 mμ in diameter. *Horizontal line* represents 0.1 μ. Approximately × 70,000

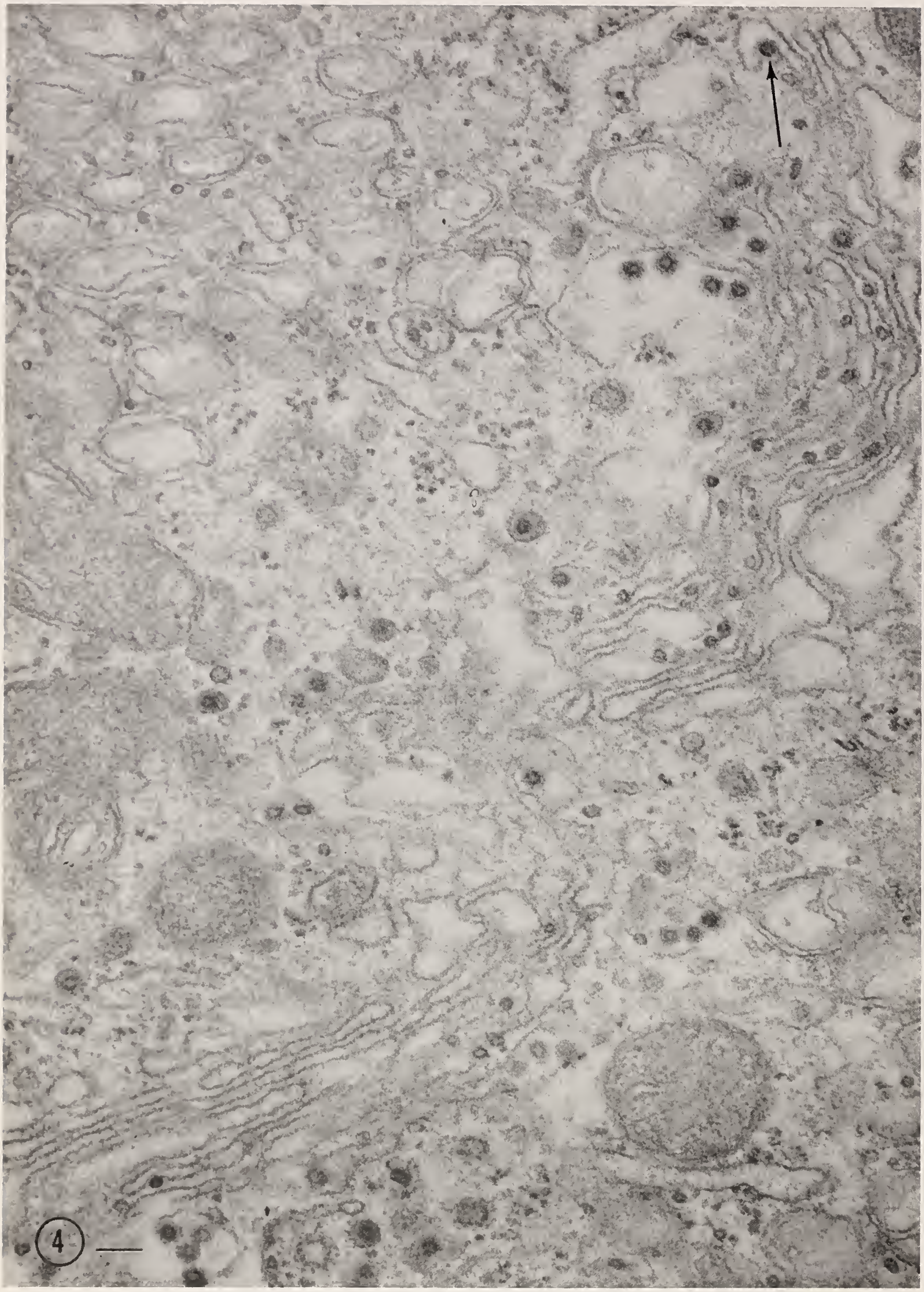


FIGURE 4.—Another area of the Golgi complex of a cell similar to that in figure 2. Small (30 mμ) electron-dense particles are in the cytoplasmic matrix between the flattened sacs. Several complete particles are within Golgi vacuoles. One particle (*arrow*) appears to be building into a vacuole. *Horizontal line* represents 0.1 μ. Approximately × 70,000

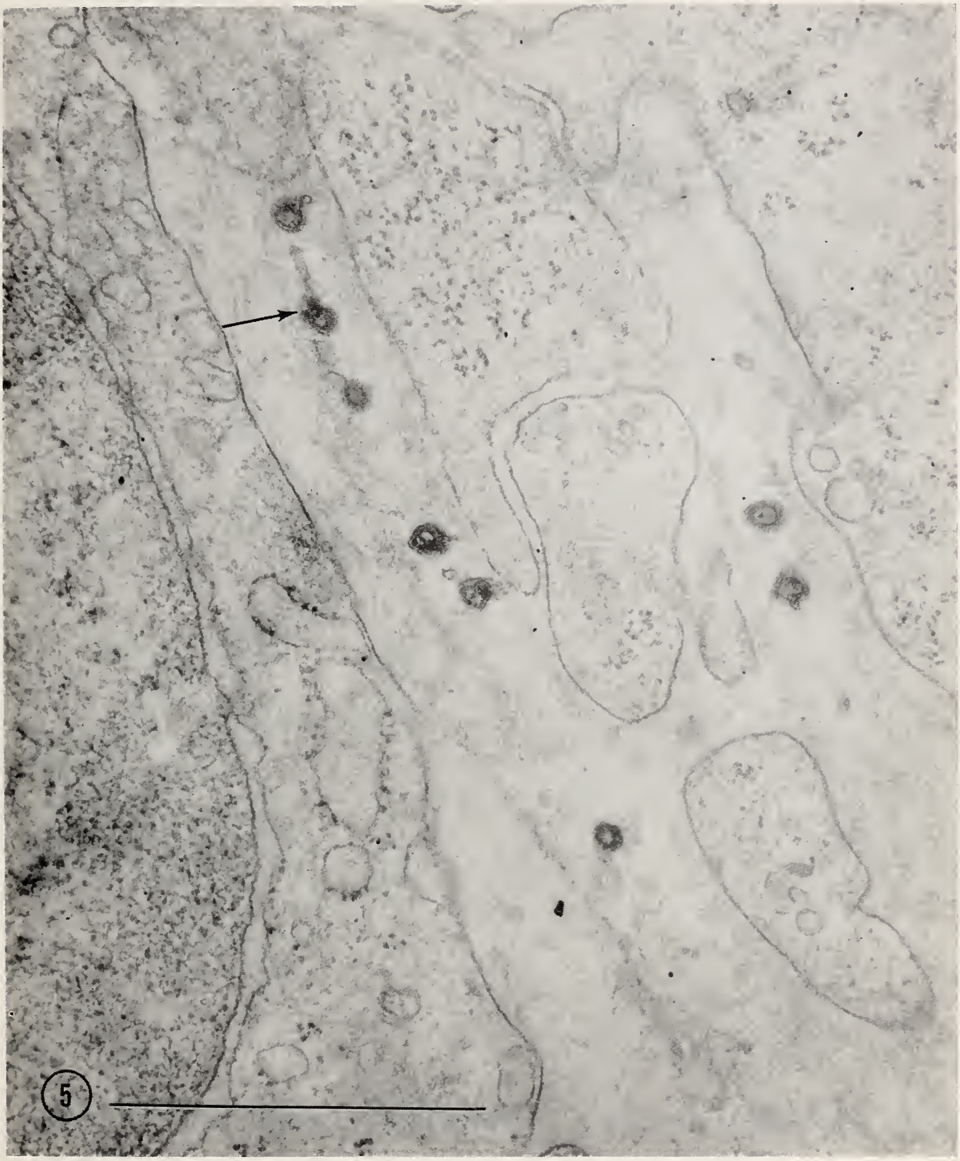


FIGURE 5.—Mature particles, one with a tail (*arrow*), indistinguishable from murine leukemia virus particles, are present between tumor cells. *Horzontal line* represents 0.1 μ. Approximately × 70,000

PLATE 27

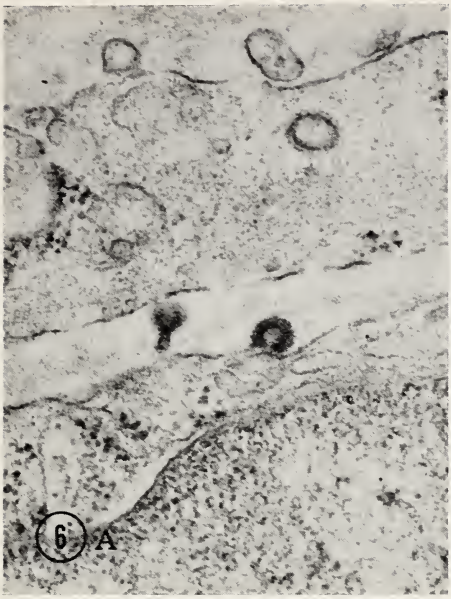
In each photograph, *horizontal line* represents 1.0 μ.

FIGURE 6.—A. Surface of a tumor cell showing a particle budding from the plasma membrane. *B.* Another area of the surface of the same cell as that in figure 6 A showing a mature particle in the process of being taken into the cell in a forming pinocytotic vesicle. *Horizontal line* represents 1.0 μ. Approximately × 52,500

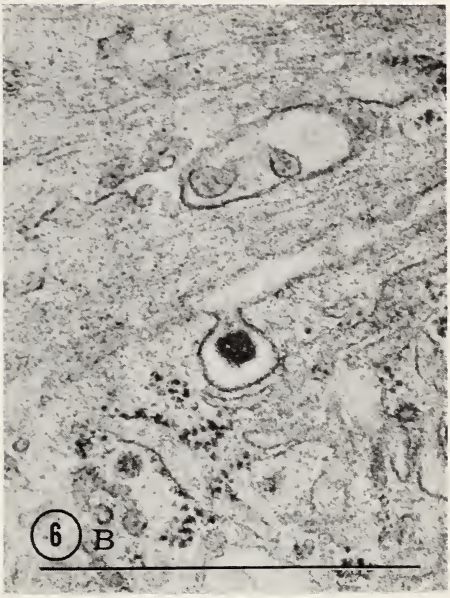
FIGURE 7.—An area of cytoplasm of a tumor cell showing a particle budding at the tip of a long stalk into a cytoplasmic vacuole. The membrane of the vacuole is differentiated in a manner similar to that of the membrane of a pinocytotic vesicle. *Horizontal line* represents 0.1 μ. Approximately × 70,000

FIGURE 8.—A viral bud at the surface of a tumor cell. The forming outer and intermediate membranes and border of the nucleoid are evident. *Horizontal line* represents 0.1 μ. Approximately × 140,000

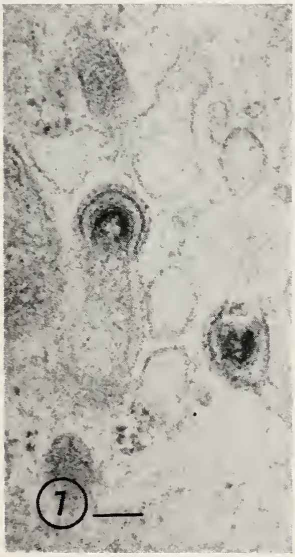
FIGURE 9.—An immature particle near the border of a tumor cell. A tail-like projection, an intermediate membrane and a nucleoid with a relatively electron-lucent central area are present. *Horizontal line* represents 0.1 μ. Approximately × 140,000



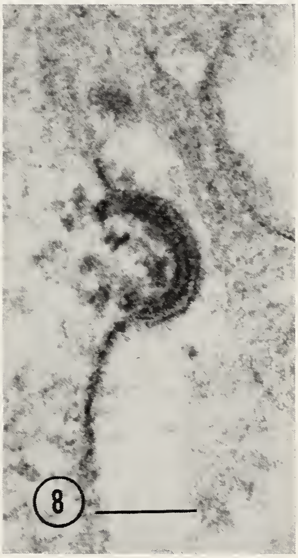
⑥ A



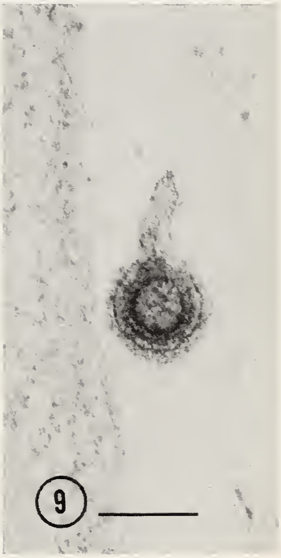
⑥ B



⑦



⑧



⑨

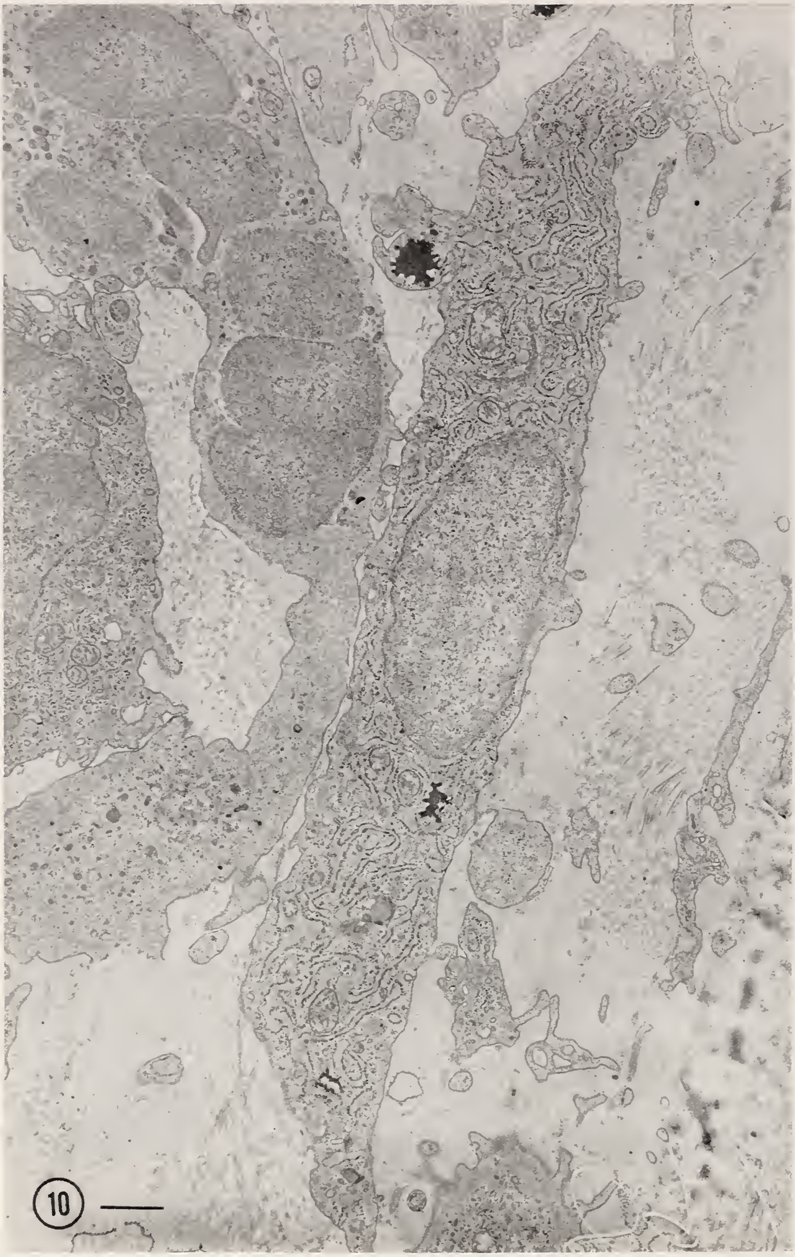


FIGURE 10.—A spindle-shaped tumor cell of type 1 at low magnification. (Cells to *left* are part of a small blood vessel.) The elongated nucleus, mitochondria with mostly circular or oval profiles, and ergastoplasm showing an orientation along the long axis of the cell are evident. *Horizontal line* represents 1.0 μ. Approximately × 8,800

11

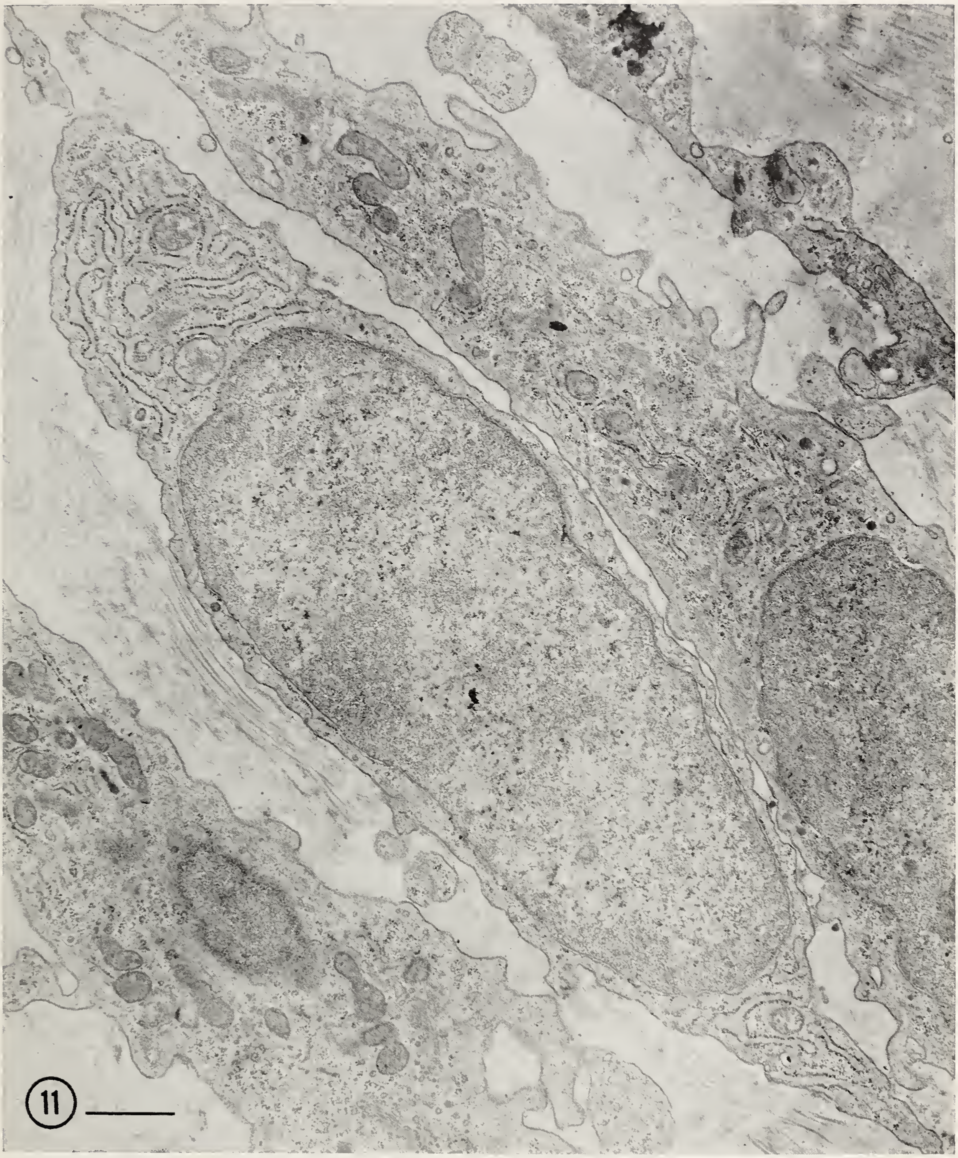


Figure 11.—Parts of 4 tumor cells of type 1 showing the same characteristics as the cell in figure 10. Mitochondria with elongated profiles are visible in two of the cells. *Horizontal line* represent 1.0 μ. Approximately × 12,500

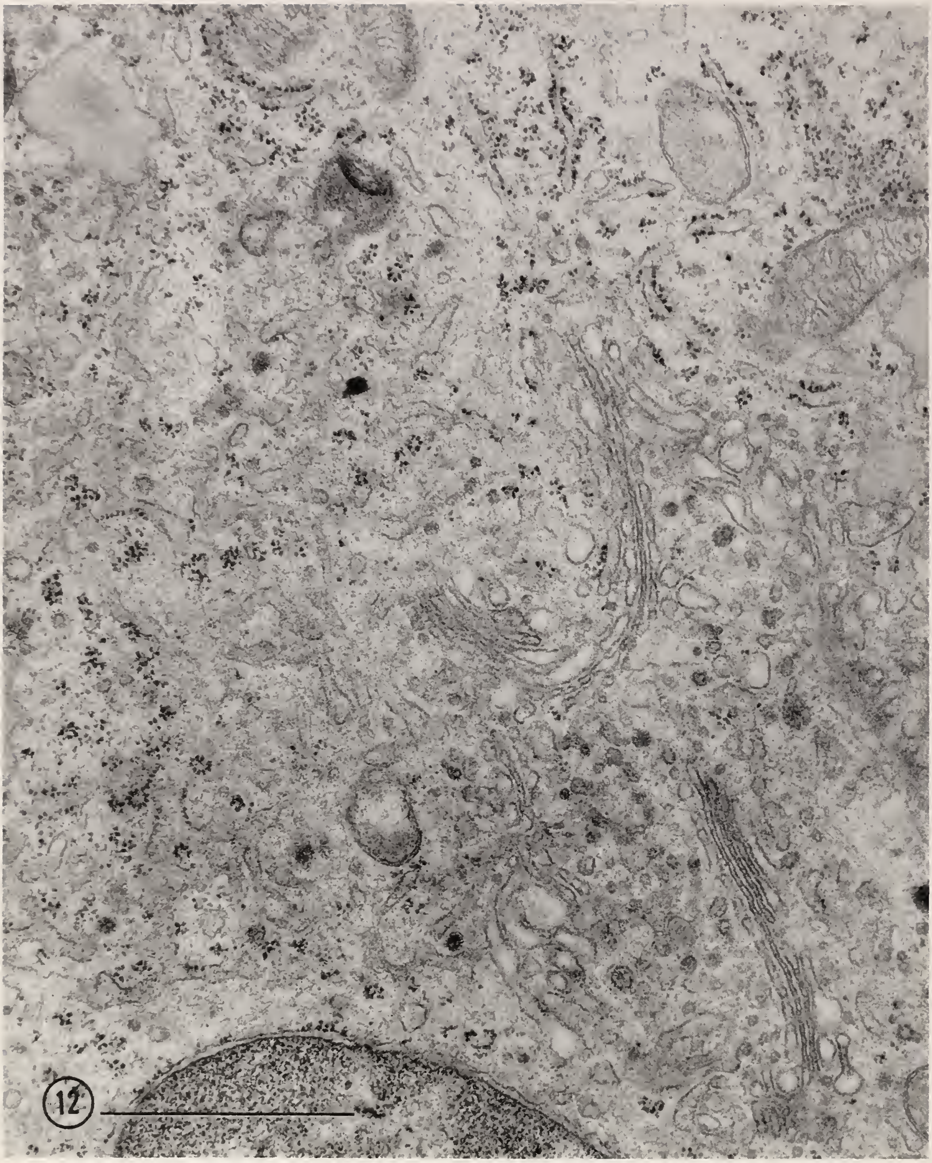
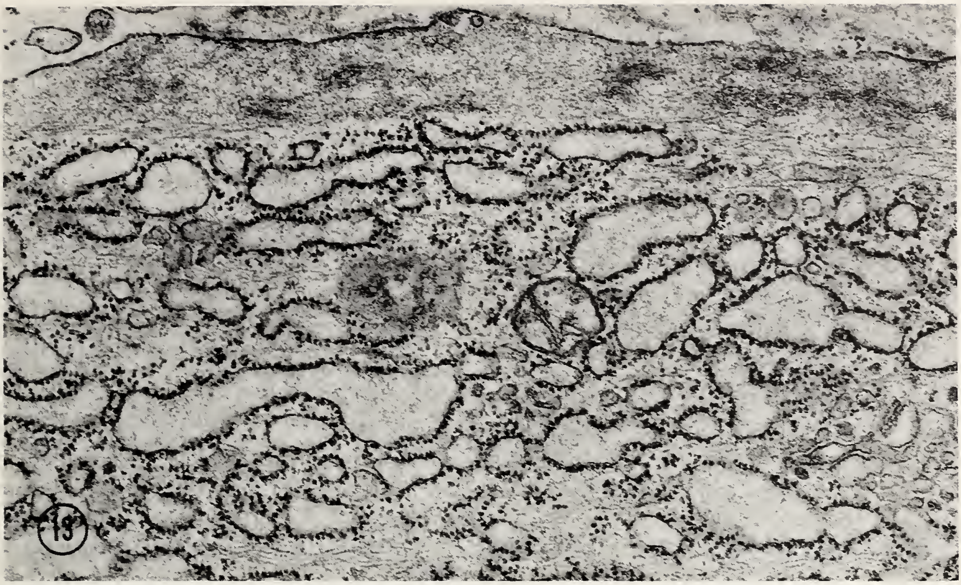


FIGURE 12.—An area of the cytoplasm of a type I tumor cell showing a characteristic Golgi complex. Flattened sacs and small vesicles are numerous but the vacuoles are not well developed. *Horizontal line* represents 1.0 μ. Approximately × 35,000

FIGURE 13.—Part of the cytoplasm of a type 1 tumor cell showing a flocculent, granular precipitate in the cisternae of the endoplasmic reticulum. Fibrils are in the cytoplasmic matrix and a bundle of fibrils intermingled with a granular substance is evident at *border* of cell. Approximately × 35,000



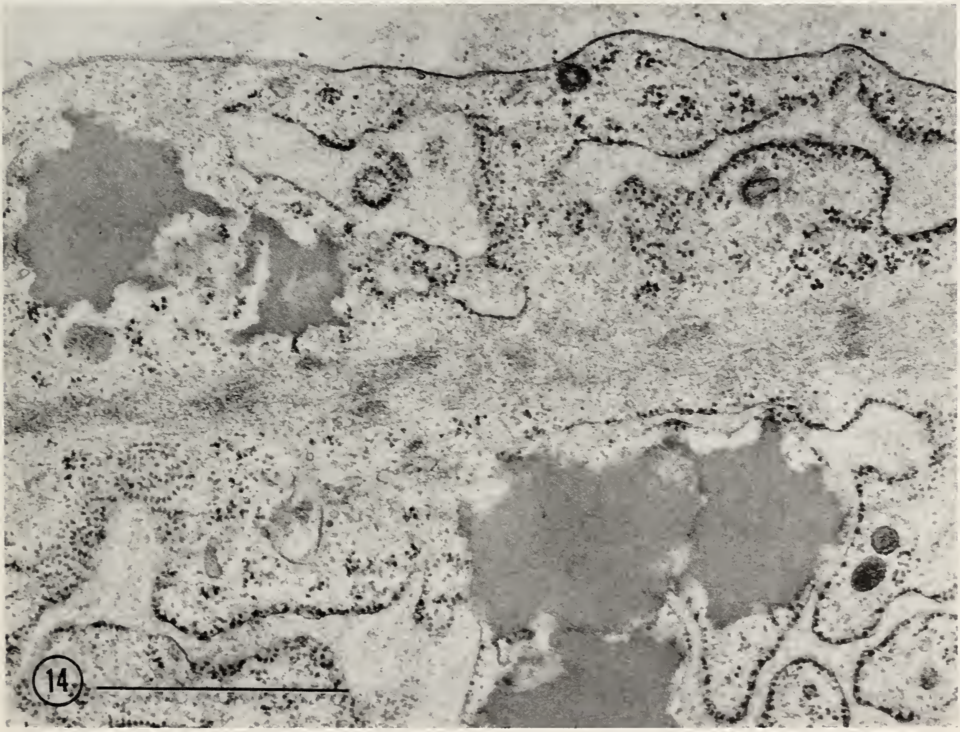


FIGURE 14.—Part of cytoplasm of a type 1 tumor cell showing a bundle of fibrils coursing through the midportion of the cell. Electron-dense areas of the bundle alternating with relatively electron-lucent areas give suggestion of periodicity. Five lipid droplets are in evidence. *Horzontal line* represents 1.0 μ. Approximately × 35,000

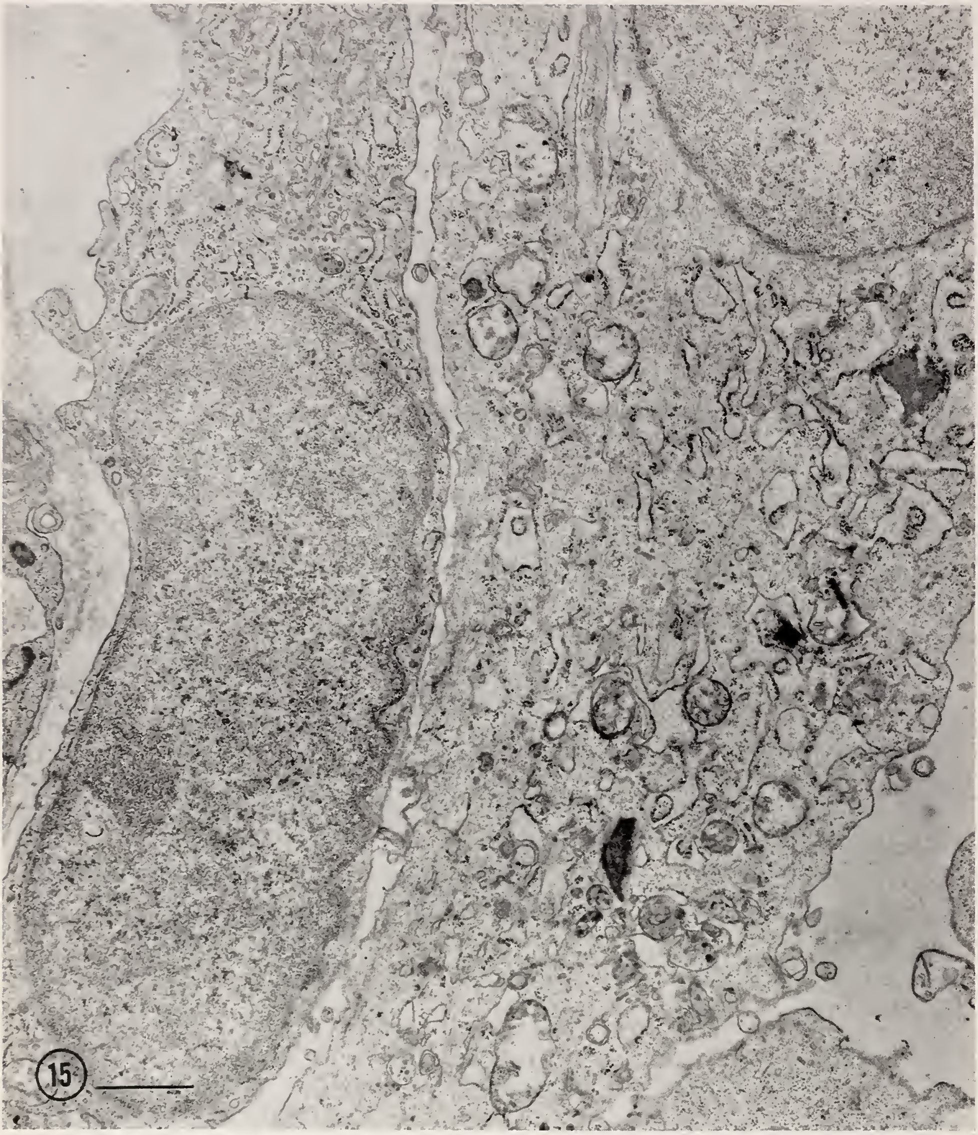


FIGURE 15.—Parts of two tumor cells, the one at *left* type 1, one at *right*, type 2. Differences in size of the cells and organization of the ergastoplasm are obvious. *Horizontal line* represents 1.0 μ. Approximately × 13,500

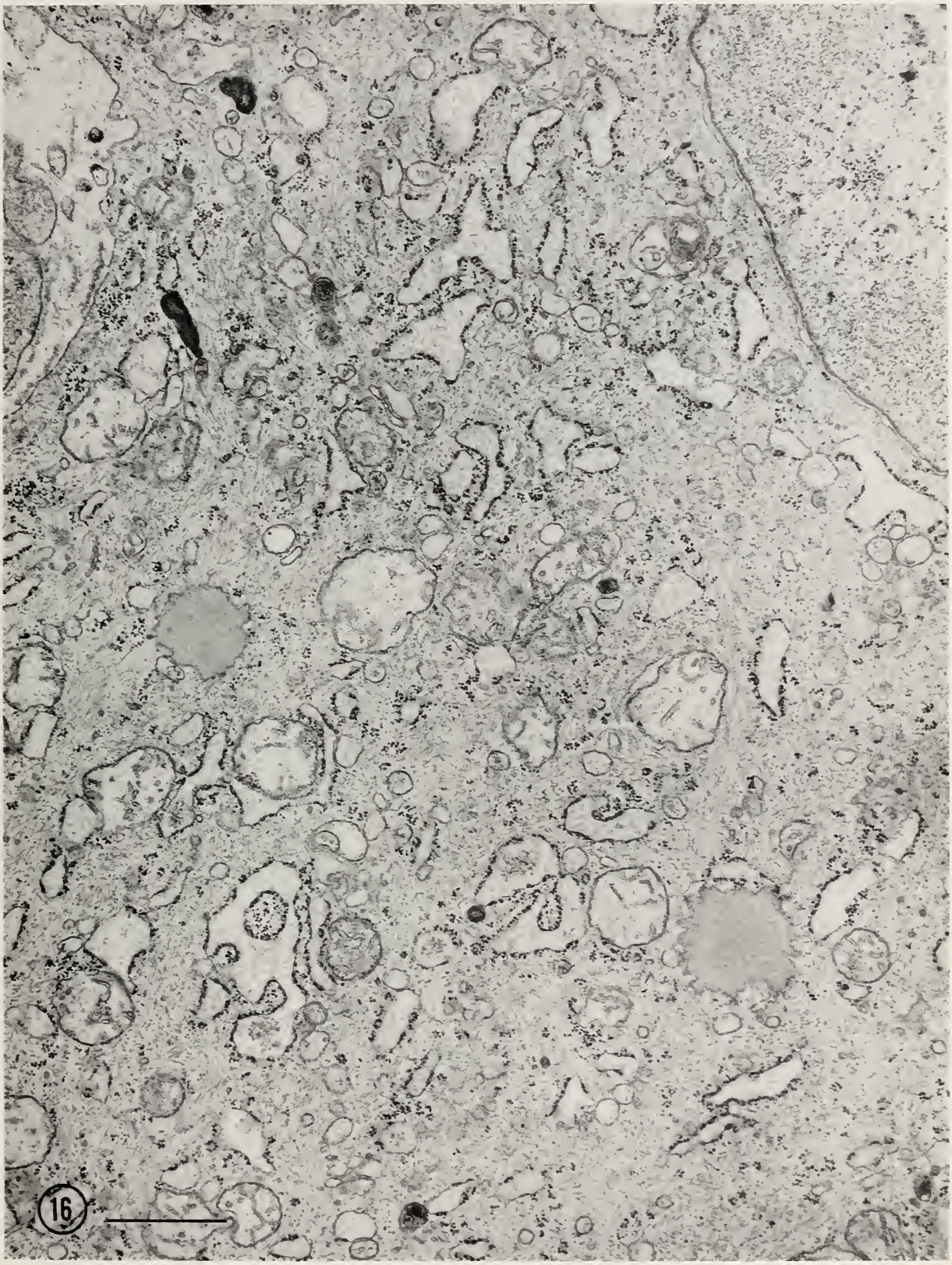


FIGURE 16.—Part of the cytoplasm and a margin of the nucleus of a type 2 tumor cell. The matrix of the mitochondria is relatively electron lucent. Fibrils are present throughout the cytoplasmic matrix. *Horizontal line* represents 1.0 μ. Approximately × 17,500

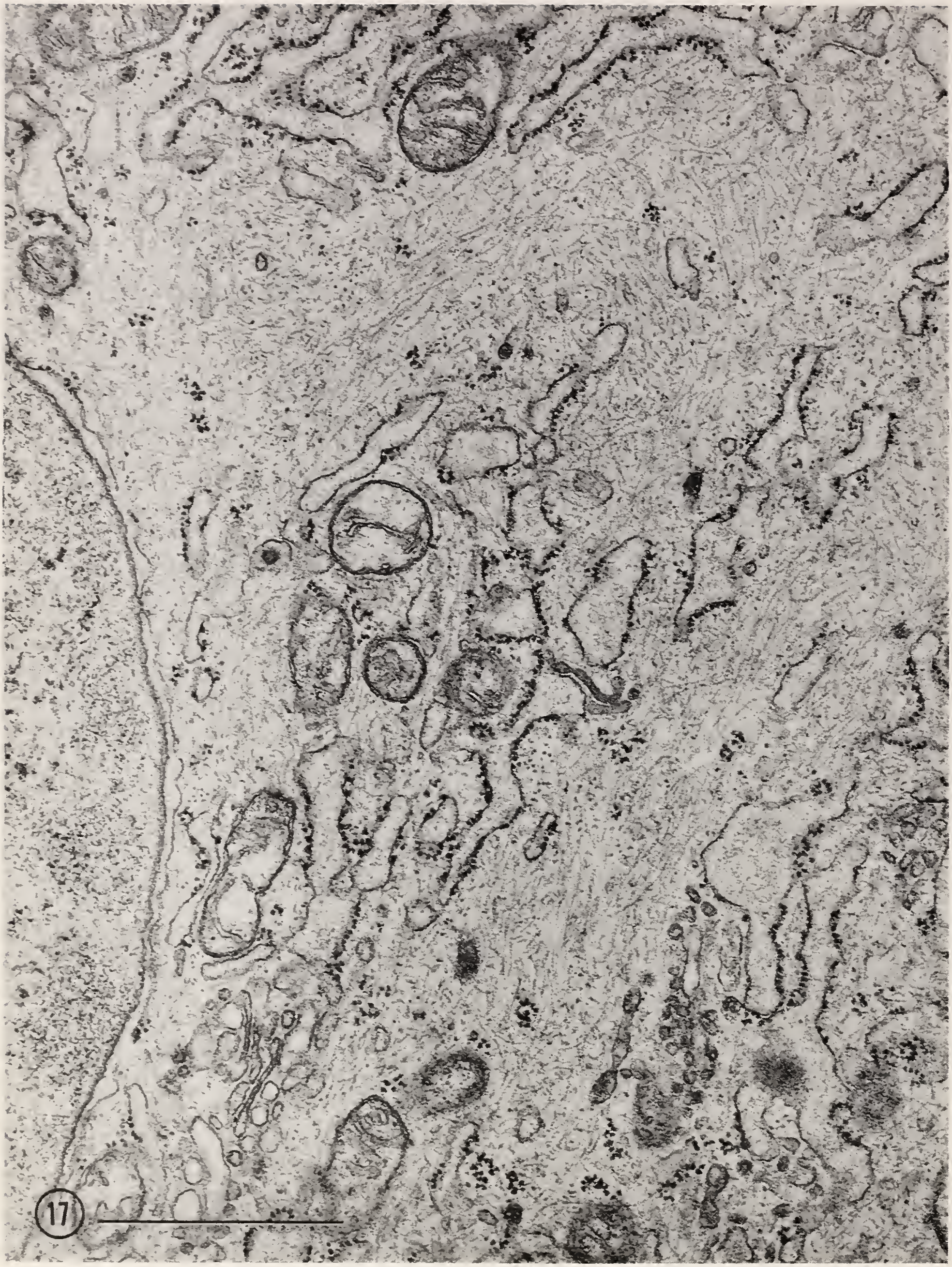


FIGURE 17.—Part of the cytoplasm and nucleus of a type 2 tumor cell. Fibrils occupy much of the cytoplasmic area. *Horizontal line* represents 1.0 μ. Approximately × 35,000

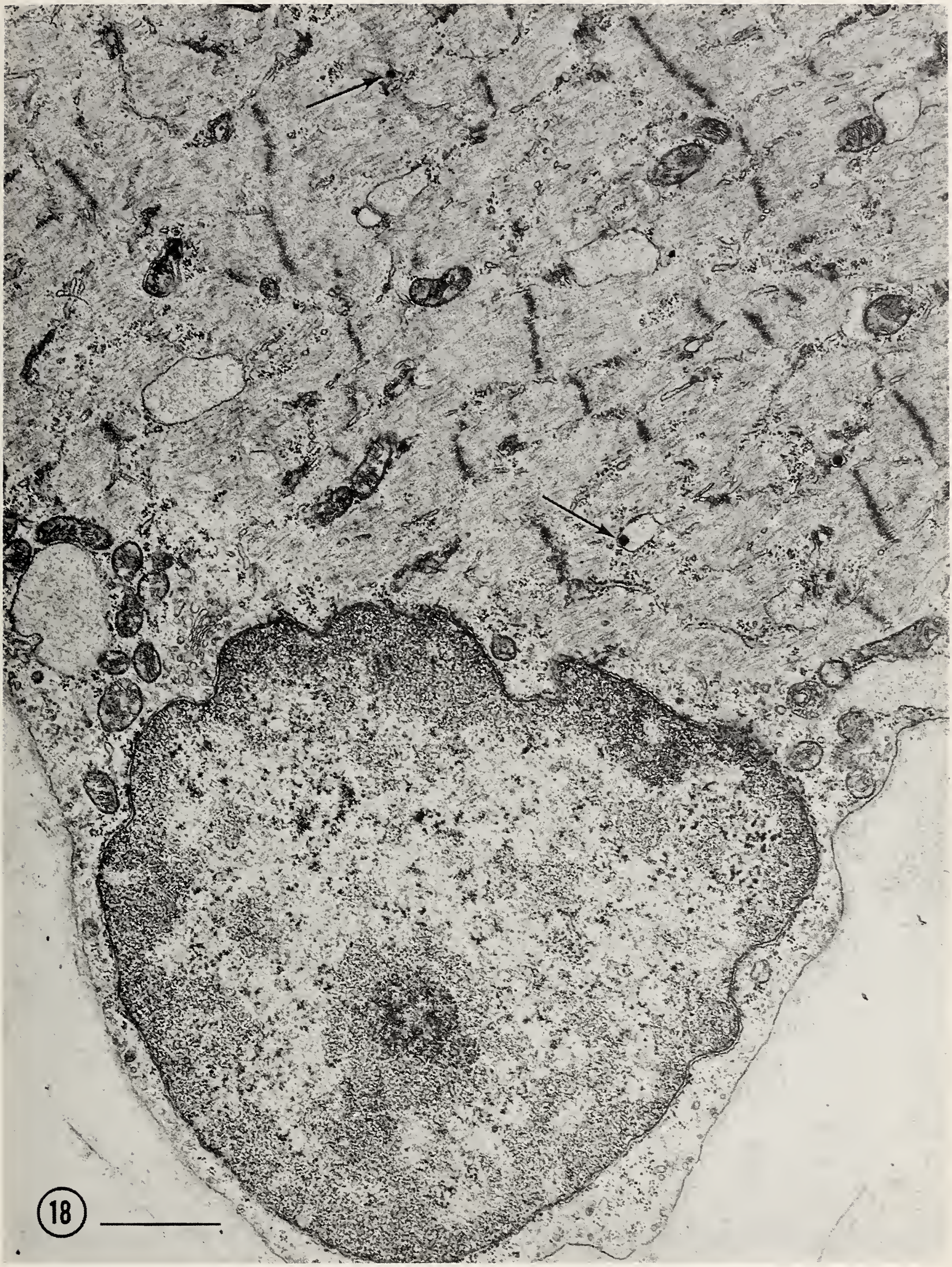


FIGURE 18.—Part of an isolated skeletal muscle cell. The nucleus with its associated undifferentiated sarcoplasm containing mitochondria and a Golgi complex are evident. "Z" lines are evident in the myofibrils, and small, dense bodies (*arrows*) are in vacuoles of the sarcoplasmic reticulum. *Horizontal line* represents 1.0 μ. Approximately × 17,500

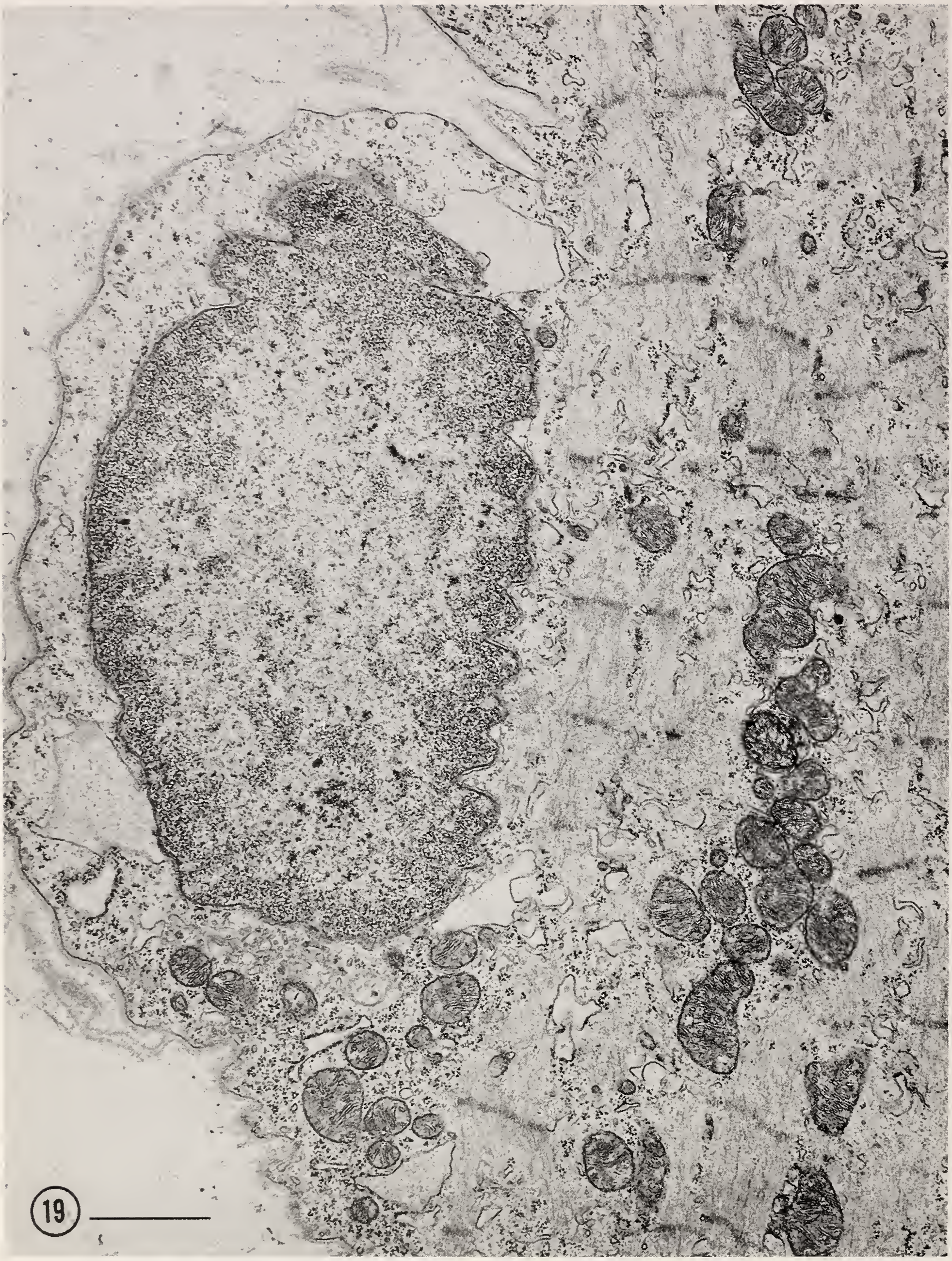


FIGURE 19.—Part of another isolated skeletal muscle cell showing a somewhat better organization of myofibrils and mitochondria. *Note* the basement membrane-like material attached to the sarcolemma. *Horizontal line* represents 1.0 μ. Approximately × 17,500

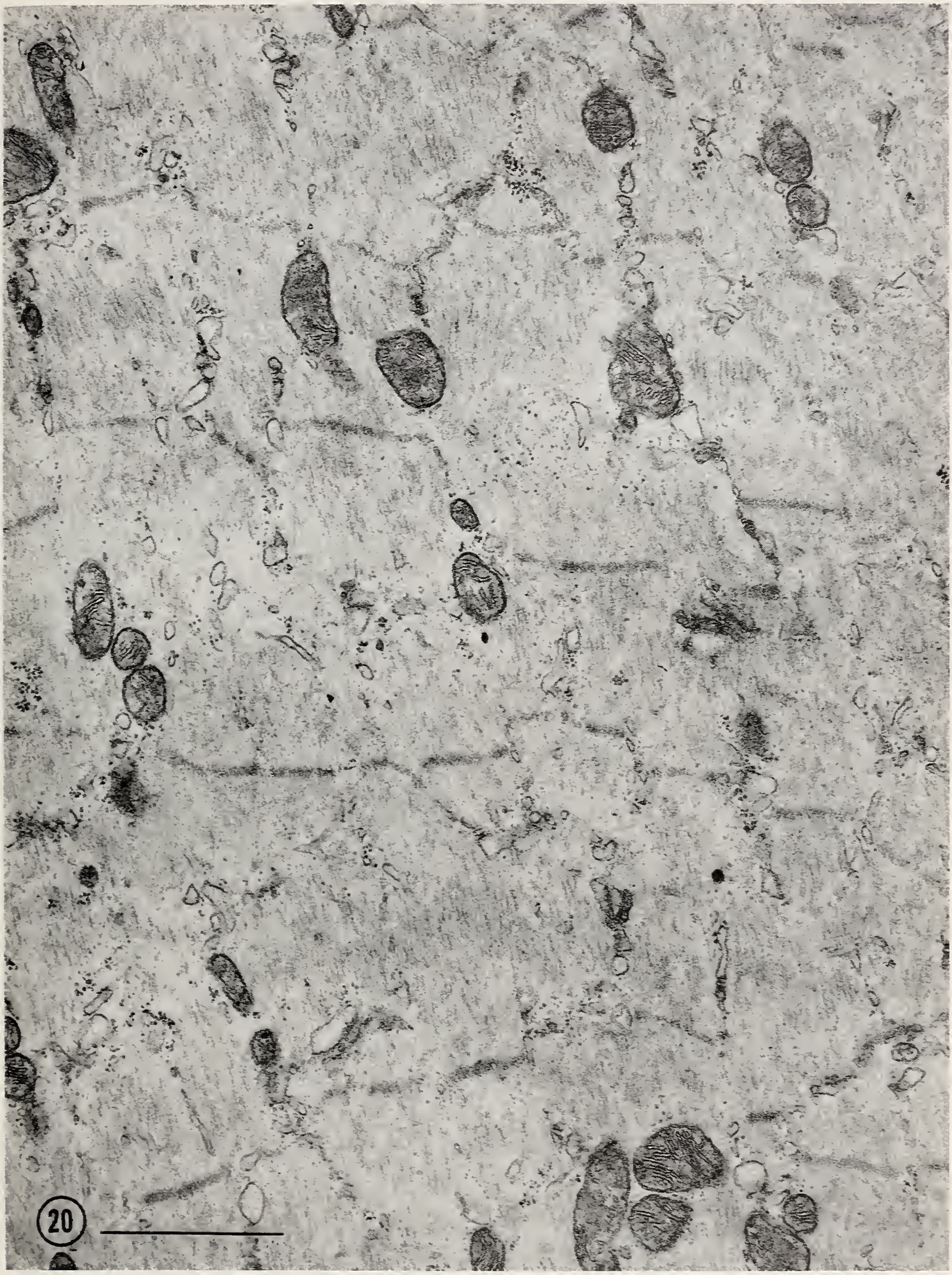


FIGURE 20.—Part of an isolated skeletal muscle cell showing more detail of the myofibrils. "Z" lines are evident, but there is no clear-cut separation into A and I bands. *Horizontal line* represents 1.0 μ. Approximately × 26,000

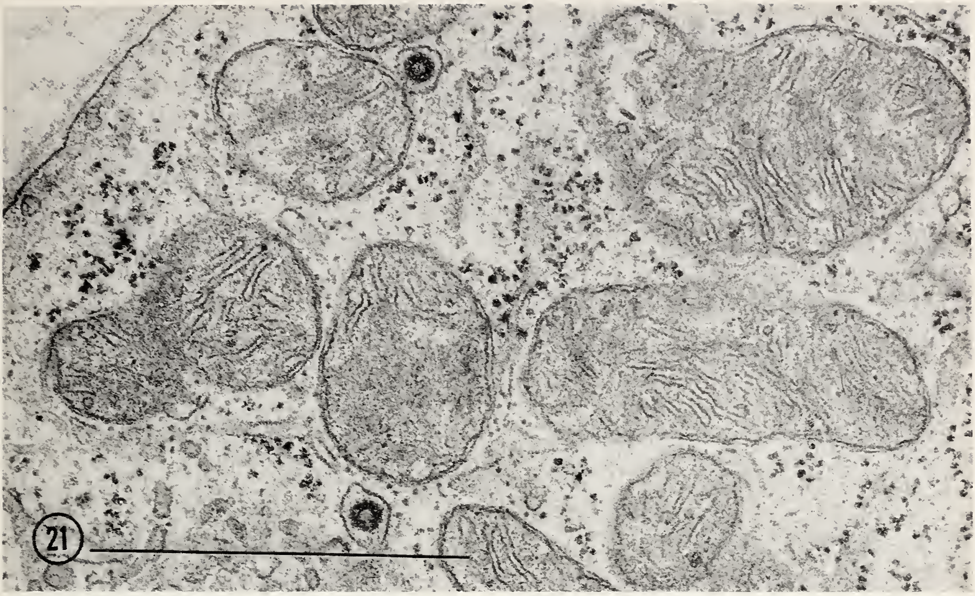
PLATE 38

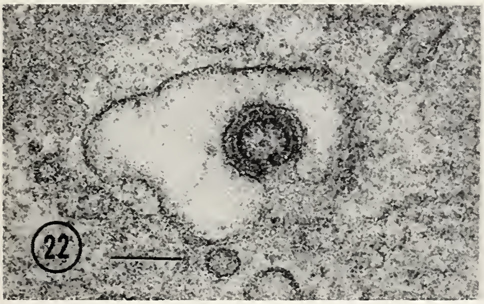
FIGURE 21.—A sarcoplasmic area of an isolated skeletal muscle cell showing detail of the mitochondria and sarcoplasmic reticulum. Two particles with the morphology of viruses (type A) are present within vacuoles of the reticulum. *Horizontal line* represents 1.0 μ. Approximately × 52,500

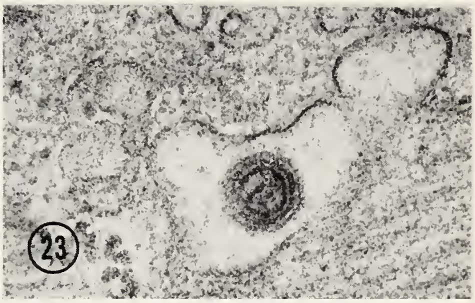
FIGURE 22.—Higher magnification of part of an isolated skeletal muscle cell showing a typical type C particle budding from the sarcolemma tubules (T system). *Horizontal line* represents 0.1 μ. Approximately × 100,000

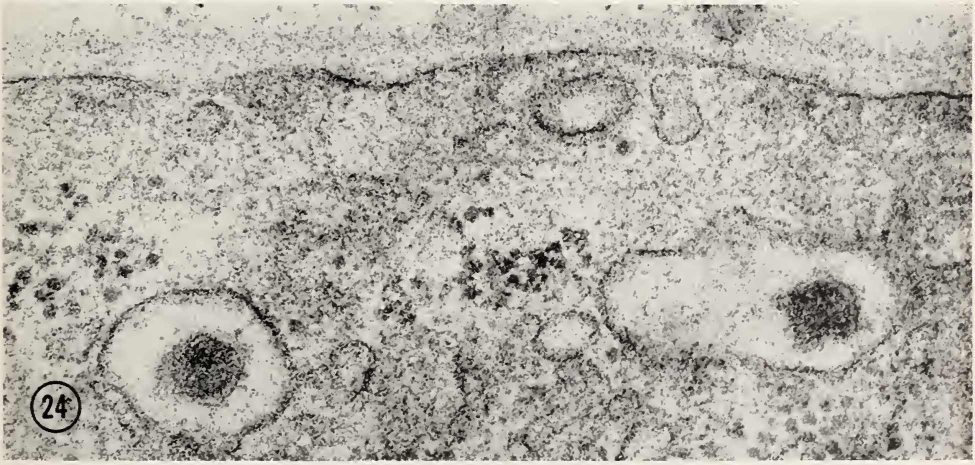
FIGURE 23.—Part of another isolated skeletal muscle cell showing an immature type C particle lying free in a cisterna of the sarcolemma tubules. Approximately × 100,000

FIGURE 24.—Part of another isolated skeletal muscle cell showing two mature type C particles in separate cisternae of the sarcolemma tubules. Approximately × 100,000


21


22


23


24

DISCUSSION

Dr. Haguenau: I imagine that everybody here is impressed with this important new fact: that a murine virus present in a leukemic viral preparation is capable of producing a solid tumor, a situation evocative of that in chicken tumors and leukemias.

Because of this resemblance I was much interested by some ultrastructural aspects of the types of cells shown here by Dr. Dalton in the murine sarcoma.

During our present electron microscopic study with Dr. H. Hanafusa and Dr. T. Hanafusa on cells infected with Rous sarcoma virus (Bryan strain) either virus-producing or non-virus-producing cells ("transformed") and cells infected with the helper virus RAV_1, we have observed striking differences in the organization of ergastoplasm. This is disrupted in transformed cells where ribosomes and polyribosomes predominate and beautifully organized in membranes when the helper alone is present.

We have wondered whether this might be an expression at the ultrastructural level of the transforming capacity of RSV (Bryan strain) a capacity absent in the helper virus RAV_1. Further work is needed to substantiate this interpretation.

Returning to the murine sarcoma, the fact that there are two types of cells, one well differentiated, one less-well differentiated, is reminiscent perhaps of a like situation and I would like to ask Dr. Dalton whether any attempt of cultivating this virus *in vitro* has been made and, if so, whether a transformation was observed.

This would perhaps be an ideal material to study defectivity in the murine leukemias.

Dr. Dalton: The cells which looked normal are the ones in which I have seen virus production—apparently a similar situation to that in the Rous. The other cell, being much larger, appears to be similar in a general sense to the transformed Rous cell, but which one is malignant or whether both are I wouldn't want to bet on right now.

Association of Enzymes: Adenosinetriphosphatase and Alkaline Phosphatase with the Virions of Murine Leukemias[1]

Guy de-Thé, *M.D.*,[2,3] *Macromolecular Biology Section, Laboratory of Viral Oncology, National Cancer Institute,*[4] *Bethesda, Maryland 20014*

THE murine as well as the avian leukemia viruses are structurally made of two different parts: a nucleocapsid and an envelope. The envelope can be removed by treatment by Tween and ether, which readily exposes the internal components or nucleocapsid. We recently studied the structure of the nucleocapsid of the Rauscher leukemia virus (RLV) after such treatment (*1*, *2*). These viruses are known to mature by a budding process through the plasma membrane of the host cells. It results that the viral envelope derives from the cell membrane. We have shown that the enzyme (adenosinetriphosphatase) associated with the avian myeloblastosis virus is localized at the viral envelope and originates at the cell membrane of the virus-producing cells (*3*). Comparable enzyme is also present at the murine leukemia virus, but appears to be different in origin (*4*).

The present study reviews the data concerned with the nucleoside phosphatase activity associated with the murine leukemia virus, and reports the presence of an alkaline phosphatase activity at the virions of the leukemic mice injected with Moloney leukemia virus (MLV).

MATERIALS AND METHODS

Tenfold concentrations of virus were prepared from viremic plasma of BALB/c mice inoculated with MLV or RLV (*5*, *6*) kindly provided by Dr. J. B. Moloney and Dr. F. J. Rauscher of the National Cancer Institute. After a short preliminary centrifugation at 7,000 to 10,000 rpm, the supernatant was spun at 22,500 rpm for 1 hour in a Spinco centrifuge with an SW39 rotor. The high-speed centrifugation pellet was fixed with either 2.5 to 5% glutaraldehyde (*7*) or 3% paraformaldehyde (trioxymethylene), buffered with sodium cacodylate at *p*H 7.4. After fixation for 30 to 60 minutes, the pellets were put in cacodylate buffer for 1 to 24 hours.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Visiting Scientist, *present address:* Centre de Recherches sur la Cellule Normale et Cancereuse, B.P. No. 8, Villejuif (Seine) France.

[3] I wish to express my gratitude to Dr. J. B. Moloney and Dr. F. J. Rauscher of the National Cancer Institute for their courtesy in providing most of the material used in this study. I greatly appreciate the excellent technical assistance of Mr. Lorenzo Waters and the competent assistance of Miss Barbara Grochowski in photography.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Various tissue specimens from viremic or leukemic animals were also examined: bone marrow, spleen, thymus, lymph nodes, and blood platelets. They were fixed with 5% glutaraldehyde or with 3% paraformaldehyde for 2 to 4 hours. After rinsing in cacodylate buffer for 2 to 15 hours, 40 to 50 μ frozen sections were made and incubated in the different cytochemical media.

The nucleoside diphosphatase and triphosphatase activities were tested by use of the Wachstein and Meisel medium at *p*H 7.2 (*8*) to which 5% sucrose was added. The substrates used were most of the diphosphonucleotides, and triphosphonucleotides, and β-glyceromonophosphate. The reaction was carried out at 37° C for 30 to 60 minutes. The insoluble lead phosphate was clearly visible under the electron beam. The alkaline phosphatase activity was more difficult to study by electron microscopy and numerous techniques were tried (*9*). The most reliable one was the Gomori technique (*10*), with β-glycerophosphate as substrate, which was modified by 1) addition of 5% sucrose in the medium and 2) passage of the incubated sections through a 2% lead nitrate solution, after a careful rinsing in 7.5% sucrose solution. The calcium phosphate formed during the reaction was therefore transformed into lead phosphate, which could be seen in the electron microscope. However, there was some diffusion of the reaction product. After a further careful rinsing, the sections were mounted in the special specimen holders (*11*), postfixed for 20 minutes with 1% osmium tetroxide containing 5% sucrose, dehydrated, and embedded in Epon-araldite (*12*). Ultrathin sections were made with an LKB microtome with a diamond knife, and examined with a Siemens I and IA electron microscope *before* and *after* being stained with uranyl acetate (*13*) and/or lead citrate (*14*).

RESULTS

Figure 1 shows the homogeneity of the virus pellets obtained from viremic mice injected with RLV. Practically no cell debris was present.

Nucleotide Phosphatase Activity

Figure 2 shows the results obtained when adenosine triphosphate (ATP) was used as substrate. Most MLV virions had a fine lead phosphate precipitate at their envelope, while RLV virus pellets consistently showed less positive virions (fig. 3). The specificity of the phosphatase associated with the viral envelope has been extensively studied (*4*). After fixation with paraformaldehyde, which preserved the nucleoside phosphatase better than glutaraldehyde, the enzyme proved to be very active with ATP and inosine triphosphate (ITP), less active with adenosine diphosphate (ADP) and inosine diphosphate (IDP), and inactive with all other nucleoside triphosphates and diphosphates and with β-glycerophosphate, respectively, used as substrates. Figure 4 shows no reaction product when uridine triphosphate (UTP) was used as substrate. Preliminary experiments with Friend leukemia virus (FLV) showed results similar to those obtained with RLV.

The nucleosidephosphatase activity (mostly adenosinetriphosphatase) of the virus-replicating organs was studied in the hope that enzymatic activity at the cell membrane, similar to that of the virus envelope, would be found. The bone marrow, spleen, thymus, lymph nodes, and platelets from the peripheral blood were examined. The following results were obtained.

Adenosinetriphosphatase activity

	Rauscher virus (RLV)	Moloney virus (MLV)
	Viremic or leukemic	Viremic or leukemic
Virus in plasma	+/++	+/−
Bone marrow Megakaryocytes	−	−
Spleen	−	−
Thymus	−	−
Lymph nodes	−	−

Megakaryocytes, recognized as the main source of virus (*15*, *16*), were extensively studied in the bone marrow and spleen. Cytoplasmic channels of the megakaryocytes (fig. 6), and those of the blood platelets (*4*) showed no adenosinetriphosphatase activity, in contrast to the peripheral cell membrane which had activity (figs. 5 and 6). In the bone marrow and spleen (fig. 7) of the viremic animals, a few scattered cells and the capillary walls were adenosinetriphosphatase-positive, while most cells were adenosinetriphosphatase-negative (fig. 7). It was very easy to find adenosinetriphosphatase-negative buds and virions (*4*), but difficult to find positive virions. Other organs likewise exhibited only adenosinetriphosphatase activity at the walls of a well-developed capillary network. Organs from normal animals reacted similarly, with a less developed capillary network.

Alkaline Phosphatase Activity

Metcalf *et al.* (*17*), Fey and Schneider (*18*), Smith (*19*), and Lagerlöf and Kaplan (*20*) described a strong alkaline phosphatase activity in the leukemic thymuses of the spontaneous leukemic AKR mice, of the Graffi virus-induced, and X-ray-induced C57BL mice, respectively. Since this enzyme appeared to be localized at the cell surface, and since we knew that leukemic cells in the thymus replicated virus by budding, it was interesting to see if virus from leukemic animals possessed alkaline phosphatase activity. Figure 8 is a section, stained for alkaline phosphatase activity, of an MLV pellet from plasma of leukemic mice. Some virions are positive (arrows) but most are negative. The former represent the proportion of virions produced in the thymus, or other organs, by alkaline phosphatase-positive cells.

In contrast, RLV pellets from viremic or leukemic animals were constantly negative for alkaline phosphatase (fig. 9). Figure 10 shows a leukemic thymus of a mouse injected with MLV. In light microscopy, large areas exhibited strong alkaline phosphatase activity. At the ultrastructural level, one could see in these areas that the precipitate was localized at the cell surface of every cell. The fine precipitate seen in the cytoplasm and nucleus of the cells is generally considered as an artifact. Occasionally, a virion was observed embedded in a thick lead phosphate precipitate (*see* inset fig. 10).

Examination of the bone marrow and spleen in leukemic animals injected with MLV showed that the megakaryoctes and associated virus particles had no activity (fig. 11), while the immature and mature leukocytes had a moderate activity (fig. 13). In these organs, the small vessels (mostly arteries) (fig. 12) were highly active. The alkaline phosphatase activity of the virus and of the organs of viremic and leukemic animals (mice and rats) injected with MLV or RLV is summarized below. The only positive animals were the leukemic mice injected with MLV (*see below*, second vertical column from the left). It can be noted that small foci of positive cells were present in the spleen and bone marrow of all virus-injected animals, and to a lesser extent in normal control animals.

Alkaline phosphatase activity

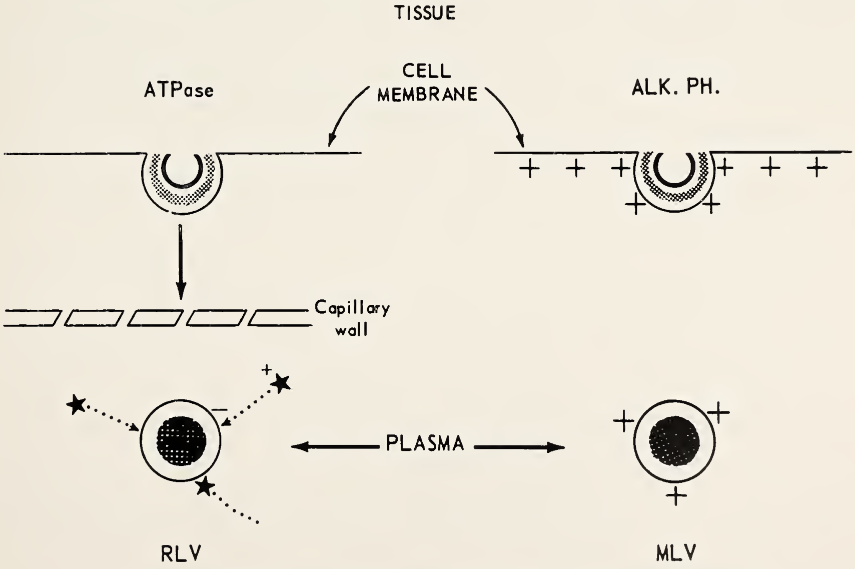
	Moloney virus (MLV)		Rauscher virus (RLV)	
	Viremic	Leukemic	Viremic	Leukemic
	Mice/rat	Mice/rat	Mice/rat	Rat
Virus in plasma	—	+	—	—
Thymus	—	+/+++	—	—
Spleen	Small foci +	+++	Small foci +	Small foci +
Bone marrow	+/++	+++	+/++	Not done
Megakaryocytes	— —	— — —	— —	Not done
Lymph nodes	Not done	+/+++	Not done	Not done

DISCUSSION

The present report shows that at least two different enzymes are associated with the envelope of the murine leukemia viruses: a nucleoside triphosphatase and an alkaline phosphatase.

The nucleoside triphosphatase is specific for ATP and ITP, in a manner similar to the enzyme associated with AMV (*3*). If AMV is taken as a model, where the origin of the viral enzyme is at the cell membrane (*3*), there is a discrepancy in the case of the adenosinetriphosphatase associated with the murine leukemia viruses: Most of the budding cell membranes do not exhibit a similar enzyme activity. Three alternatives may explain this discrepancy: 1) The proper adenosinetriphosphatase-positive and virus-replicating cells were not examined; 2) the enzymatic activity of the virus is the result of an adsorption of a plasma enzyme at the virus surface; and 3) this enzymatic activity is present, although inhibited, at the limiting membrane of the channels of the megakaryocytes. Once the virions are released in the plasma, the enzyme could become active. Text-figure 1 presents the second alternative selected as the most probable. Some data indicate that MLV, Rous sarcoma virus, AMV, and mammary tumor virus are electro-negatively charged [*see* Discussion in (*4*)]. Therefore the charged virions could attract differently charged macromolecules such as plasma enzymes. The third hypothesis implies a dynamic process that may be feasible. Experiments are actually under way to determine a possible role of this virus-associated adenosinetriphosphatase.

The alkaline phosphatase present at the envelope of some virions in MLV leukemic plasmas clearly has its origin in the alkaline phosphatase-positive leukemic cells (text-fig. 1). This enzyme thus represents an easy marker to trace the proportion of virions produced by the leukemic lymphoblasts. In all experiments the proportion was small, showing that most of the virions are made by nonleukemic cells. The presence of these



Text-figure 1.—Origin of enzymes in murine leukemia viruses.

two phosphatases at the envelope of the murine leukemia viruses should stimulate the search for various other enzymes, related or not with the cell membrane, and of their possible role in the viral infection.

SUMMARY

Phosphatase activities were found, by ultrastructural cytochemistry, to be associated with the envelope of purified Rauscher leukemia virus (RLV) and Moloney leukemia virus (MLV).

Adenosinetriphosphatase and inosinetriphosphatase activity (probably one enzyme) was present at the envelope of most RLV virions from viremic mice. Similar enzymatic activity was not found at the virus-budding membranes of the channels of the megakaryocytes nor at the cell membrane of most virus-producing cells in the bone marrow, spleen, thymus, and lymph nodes of the infected animals. The origin of the viral enzyme was therefore not clearly understood, and might be due to the adsorption of a plasma enzyme at the virus surface.

Alkaline phosphatase, an enzyme different from that described above, was also found at the surface of some virions of leukemic mice injected with MLV. In these animals the cell membrane of leukemic lymphoblasts in the thymus, spleen, and lymph nodes had a high alkaline phosphatase activity. The presence of this second enzyme at the envelope of some virions was therefore related to the presence of a similar enzyme at the virus budding cell membrane of the leukemic cells.

REFERENCES

(*1*) DE-THÉ, G., and O'CONNOR, T. E.: Structure of a murine leukemia virus after disruption with Tween-ether, and comparison with two myxoviruses. Virology 28: 713–728, 1966.

(*2*) O'CONNOR, T. E., RAUSCHER, F. J., DE-THÉ, G., FINK, M. A., and GERBER, P.: Murine leukemia viruses: Rupture with ether and detergents to subviral constituents. Nat Cancer Inst Monogr 22: 205–221, 1966.

(*3*) DE-THÉ, G., BECKER, C., and BEARD, J. W.: Virus of avian myeloblastosis (BAI strain A). XXV. Ultracytochemical study of virus and myeloblast phosphatase activity. J Nat Cancer Inst 32: 201–235, 1964. *See also:* DE-THÉ, G.: Localization and origin of the adenosinetriphosphatase activity of avian myeloblastosis virus. A review. Nat Cancer Inst Monogr 17: 651–671, 1964.

(*4*) ———: Ultrastructural cytochemistry of enzymes associated with murine leukemia viruses. I. Adenosinetriphosphatase. Int J Cancer 1: 119–138, 1966.

(*5*) MOLONEY, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J Nat Cancer Inst 24: 933–951, 1960.

(*6*) RAUSCHER, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(7) Sabatini, D. D., Bensch, K., and Barrnett, R. J.: Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol 17 : 19–58, 1963.

(8) Wachstein, M., and Meisel, E.: Histochemistry of hepatic phosphatases at a physiologic pH with special reference to the demonstration of bile canaliculi. Amer J Clin Path 27 : 13–33, 1957.

(9) de-Thé, G.: Méthode an plomb pour la mise en evidence de la phosphatase alcaline en microscopie electronique. J Microscopie 4 : 130–131, 1965.

(10) Gomori, G.: Microtechnical demonstration of phosphatase in tissue sections. Proc Soc Exp Biol Med 42 : 23–26, 1939.

(11) de-Thé, G., and Waters, L.: Technique d'inclusion pour objets minuscules on fragiles en microscopie electronique. J Microscopie. In press.

(12) Mollenhauer, H. H.: Plastic embedding mixtures for use in electron microscopy. Stain Techn 39 : 111–114, 1964.

(13) Gibbons, I. R., and Bradfield, J. R. G.: Experiments in staining thin sections for electron microscopy. *In* Proc Stockholm Conf on Electron Microscopy. New York, Academic Press Inc., 1957, pp 121–124.

(14) Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17 : 208–212, 1963.

(15) de Harven, E., and Friend, C.: Further electron microscope studies of a mouse leukemia induced by cell-free filtrates. J Biophys Biochem Cytol 7 : 747–751, 1960.

(16) Dalton, A. J.: The Moloney agent. *In* Tumors Induced by Viruses: Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York, Academic Press Inc., 1962, pp 207–217.

(17) Metcalf, D., Sparrow, N., and Wyllie, R.: Alkaline phosphatase activity in mouse lymphoma tissue. Aust J Exp Biol 40 : 215–224, 1962.

(18) Fey, F., and Schneider, E. J.: Alkalische Phosphatase bei Virus induzierten Leukamien der Maus. Acta Biol Med German 11 : 264–273, 1963.

(19) Smith, C.: Studies on the thymus of the mammal. XIII. Histochemistry of irradiated thymuses of C57BL strain mice. J Nat Cancer Inst 29 : 375–388, 1962.

(20) Lagerlöf, B., and Kaplan, H. S.: Histogenesis and alkaline phosphatase activity (AP) of radiation- and virus-induced lymphomas of C57BL mice. Fed Proc 24 (2) Pt. 1, 684, 1965.

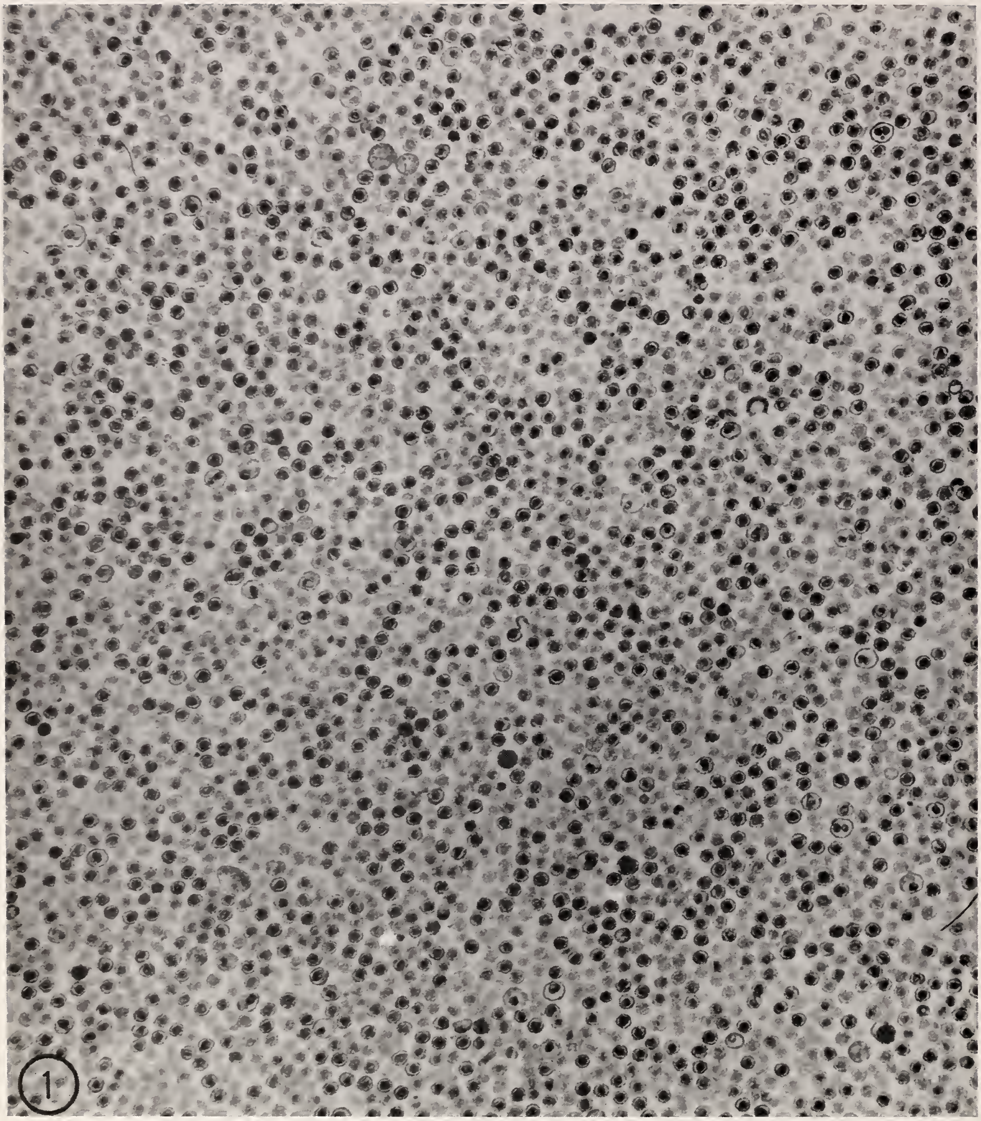


FIGURE 1.—Rauscher leukemia virus (RLV) pellet from viremic BALB/c mice. *Note* the homogeneity of the preparation. × 18,000

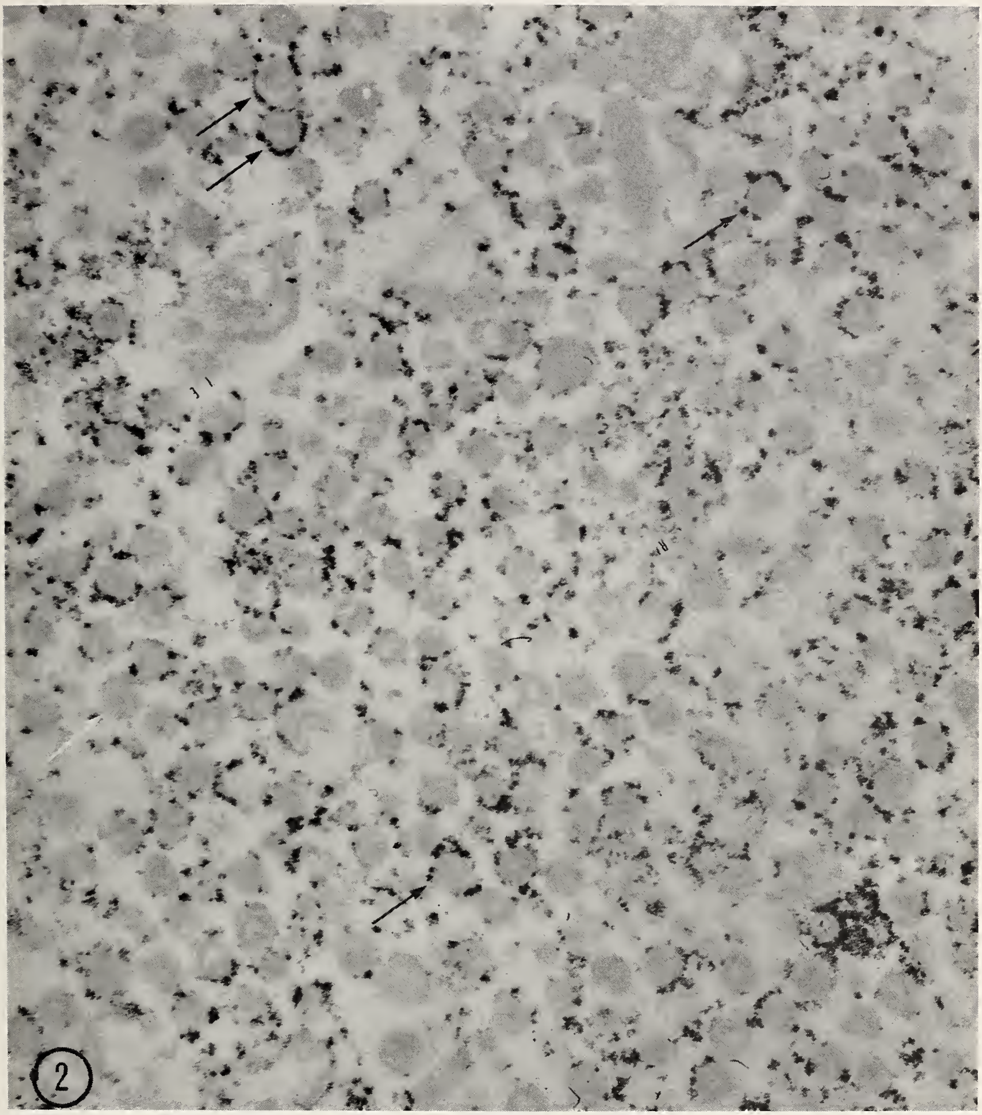


FIGURE 2.—Rauscher leukemia plasma virus incubated for adenosinetriphosphatase activity. Most virions show a fine precipitate at their surface (*arrows*), while few appear negative (*white arrow*). The thin section has not been stained for contrast; therefore, the nucleoid of the particles do not stand out as in figure 1. × 60,000

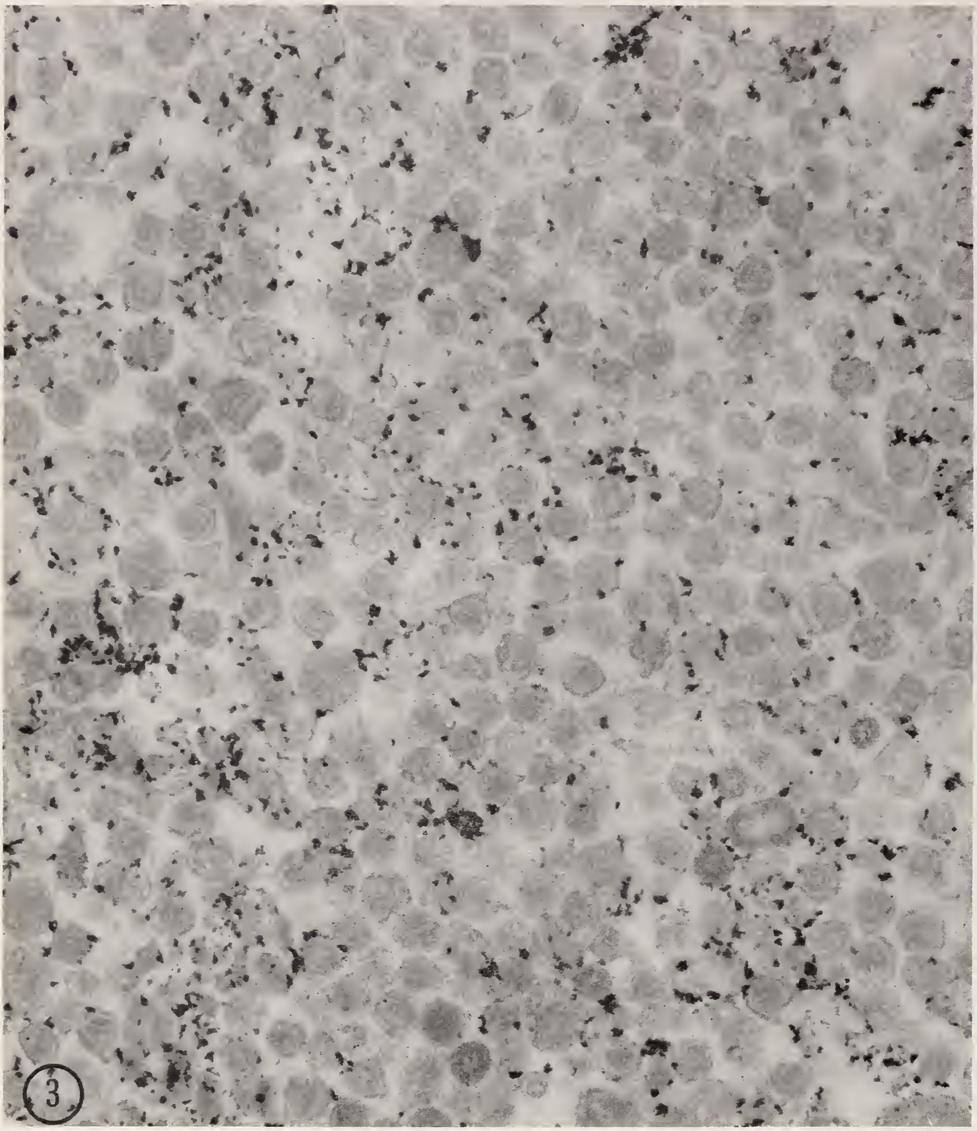


FIGURE 3.—Moloney plasma virus stained for adenosinetriphosphatase as figure 2. *Note* that only few virions are positive in contrast with Rauscher leukemia virus pellet (fig. 2). The thin section has been stained with uranyl acetate for 20 minutes. × 60,000

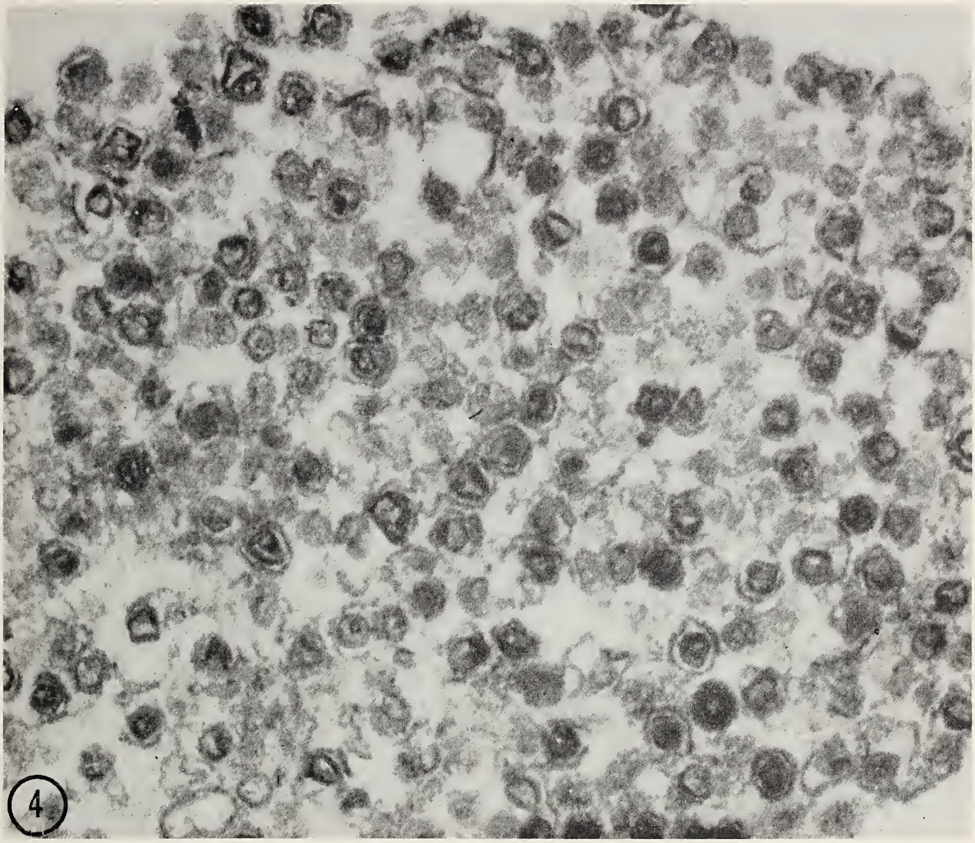


FIGURE 4.—Rauscher leukemia virus incubated with uridine triphosphate as substrate for 60 minutes. No lead phosphate precipitate can be seen about the virus. *Compare* with figures 1 and 2. Thin section stained for contrast with uranyl acetate for 45 minutes. × 65,000

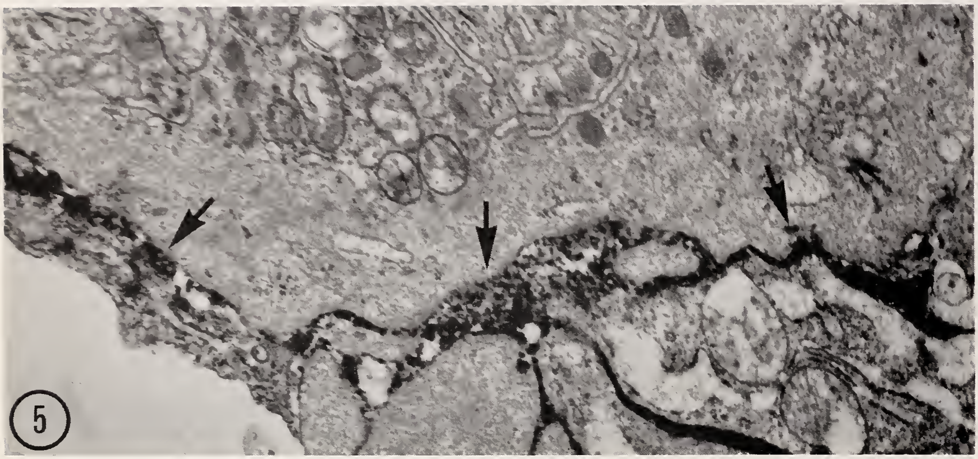


FIGURE 5.—Peripheral part of a megakaryocyte in bone marrow of a viremic mouse infected with Moloney leukemia virus. The cell membrane is strongly adenosinetriphosphatase-positive (*arrows*), while the cytoplasmic channels (*upper part*) are negative. × 21,000

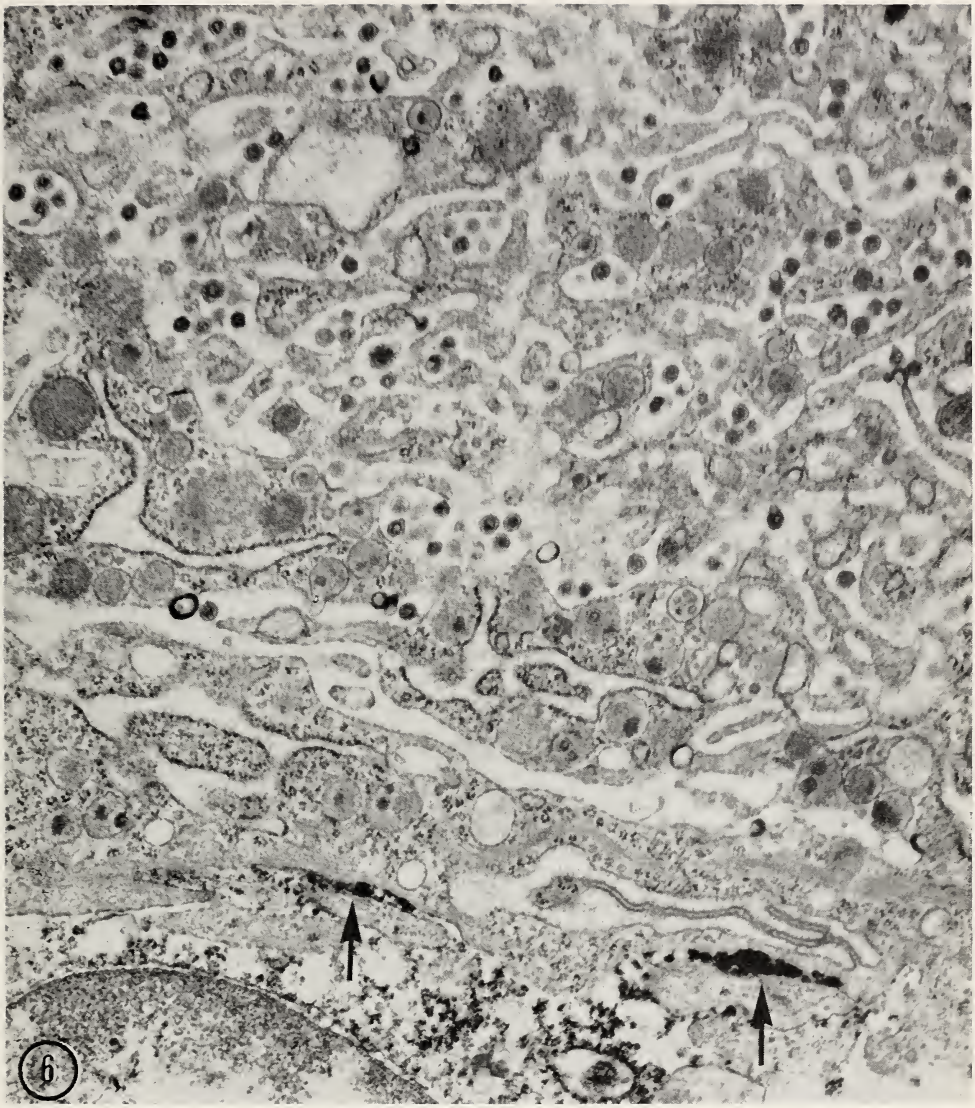


FIGURE 6.—Megakaryocyte in a large spleen of a mouse infected with Rauscher leukemia virus. *At bottom*, the peripheral cell membrane is adenosinetriphosphatase-positive (*arrows*), while the cytoplasmic channels and associated virions are entirely negative (*central* and *upper* part). × 28,000

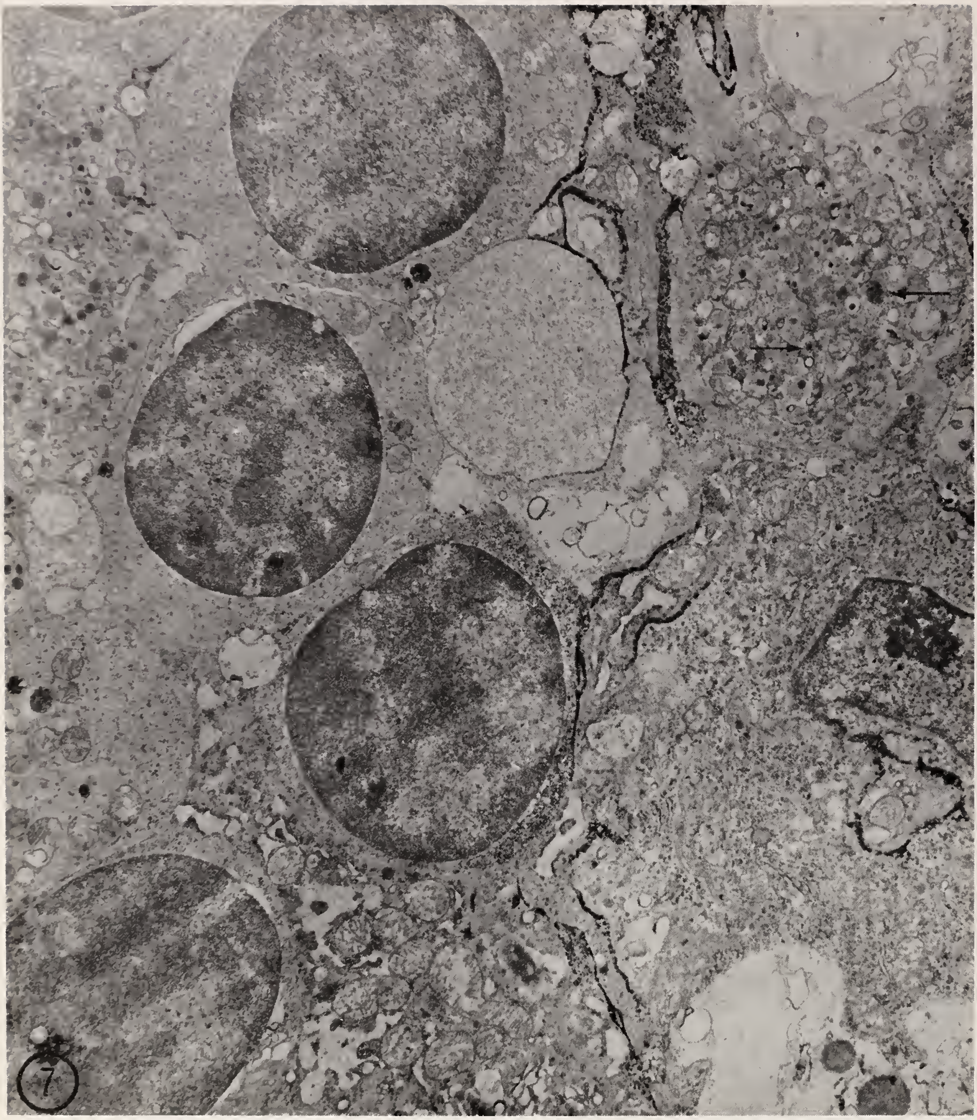


FIGURE 7.—Spleen of mouse injected with Rauscher leukemia virus. After staining for adenosinetriphosphatase activity, capillary walls are active, while proliferative cells (*left*) are negative. × 9,000

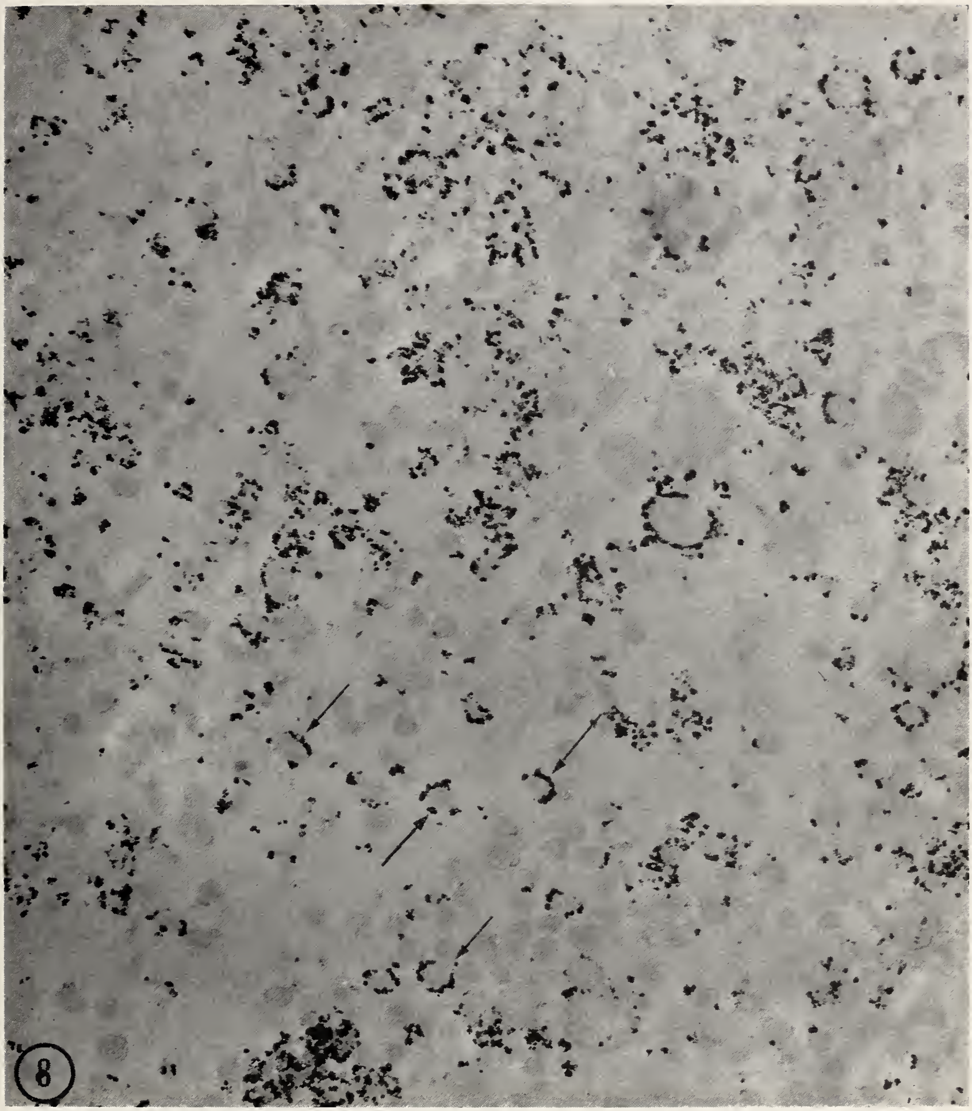


FIGURE 8.—Moloney leukemia plasma virus from leukemic mice. After incubation for alkaline phosphatase activity, some virions are positive (*arrows*) and many are negative. There is also some positive cell debris in *upper part* of micrograph. × 35,000

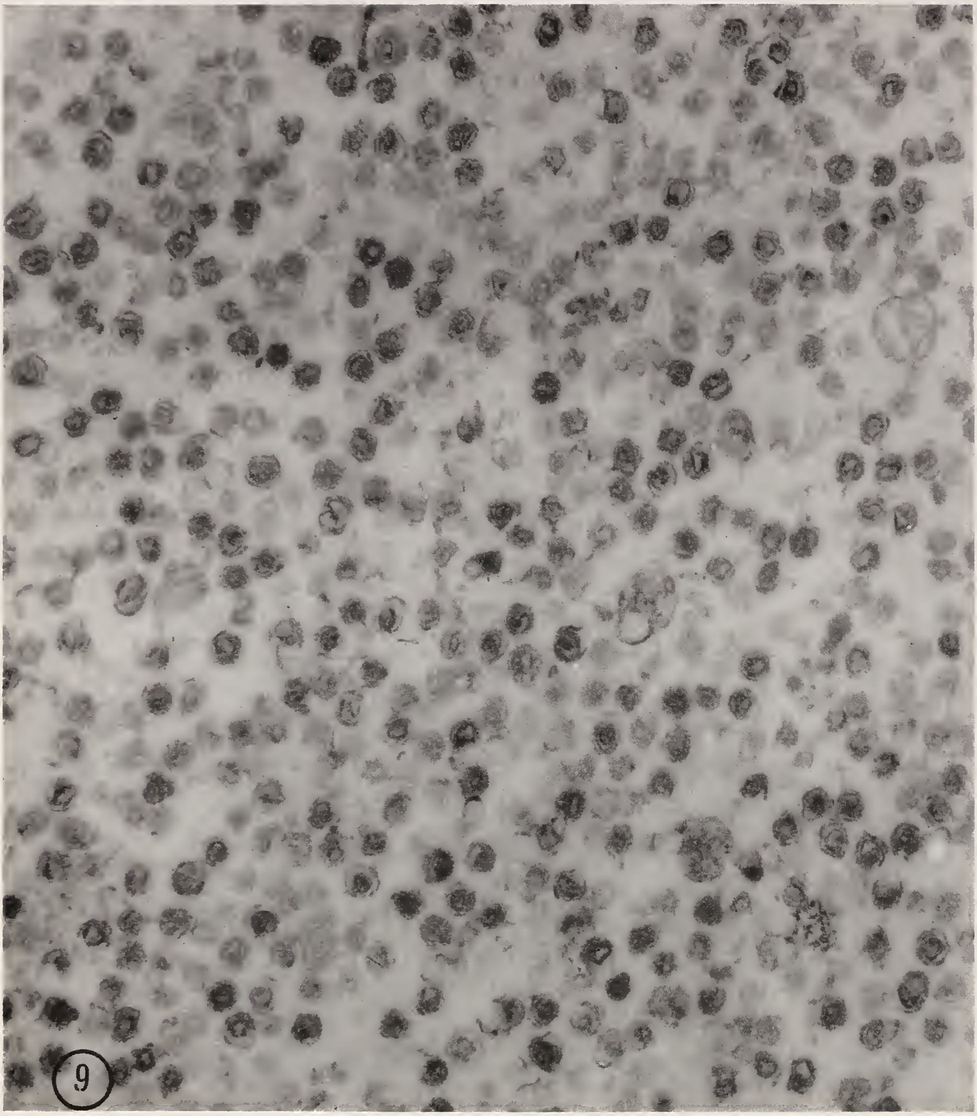


Figure 9.—Rauscher leukemia plasma virus from viremic mice. After incubation for alkaline phosphatase, no virion is active, in contrast to Moloney leukemia virus of figure 8. × 43,500

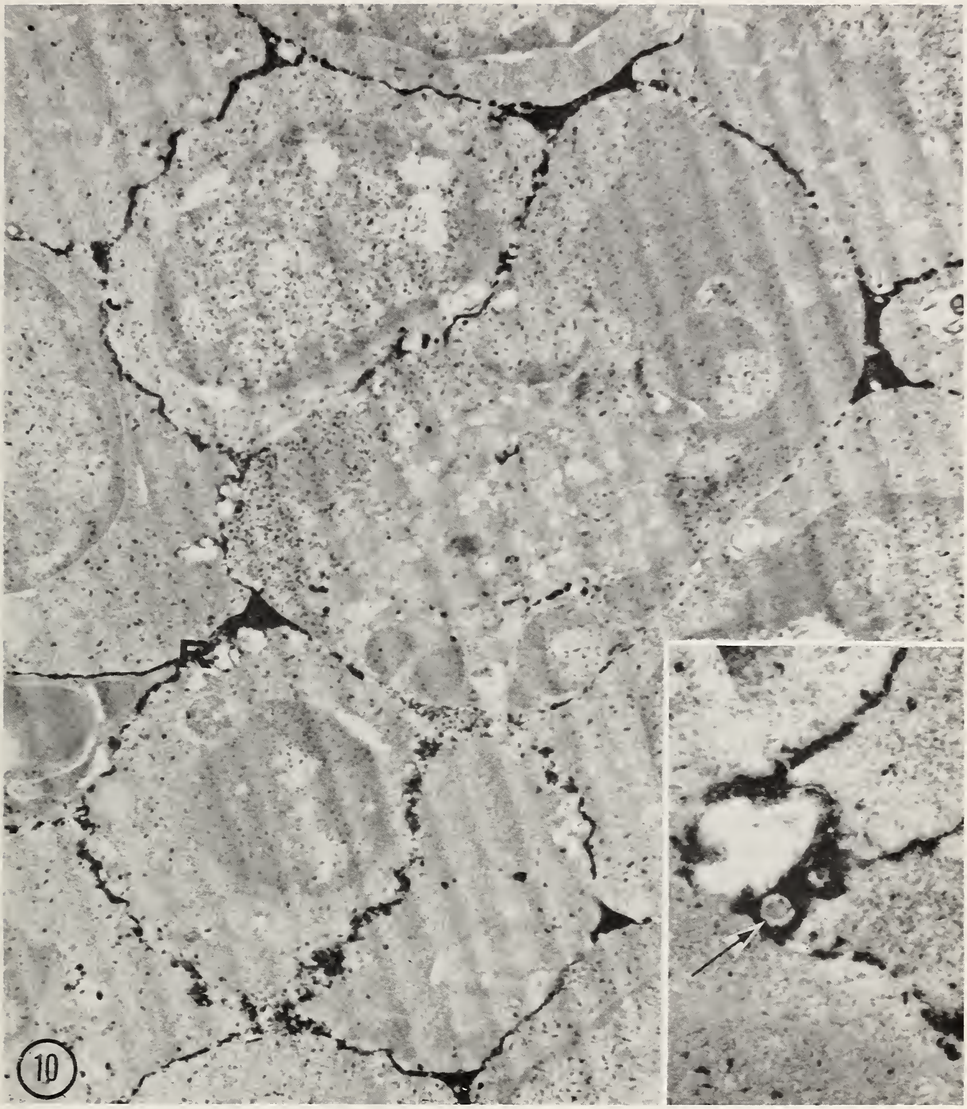


FIGURE 10.—Thymus from leukemic mice infected with Moloney leukemia virus. Leukemic cells have alkaline phosphatase activity at cell membrane. Occasionally a virus particle is embedded in the lead phosphate precipitate between cells (*inset*). Fine precipitate in cytoplasms and in nuclei is considered to be an artifact. × 12,000

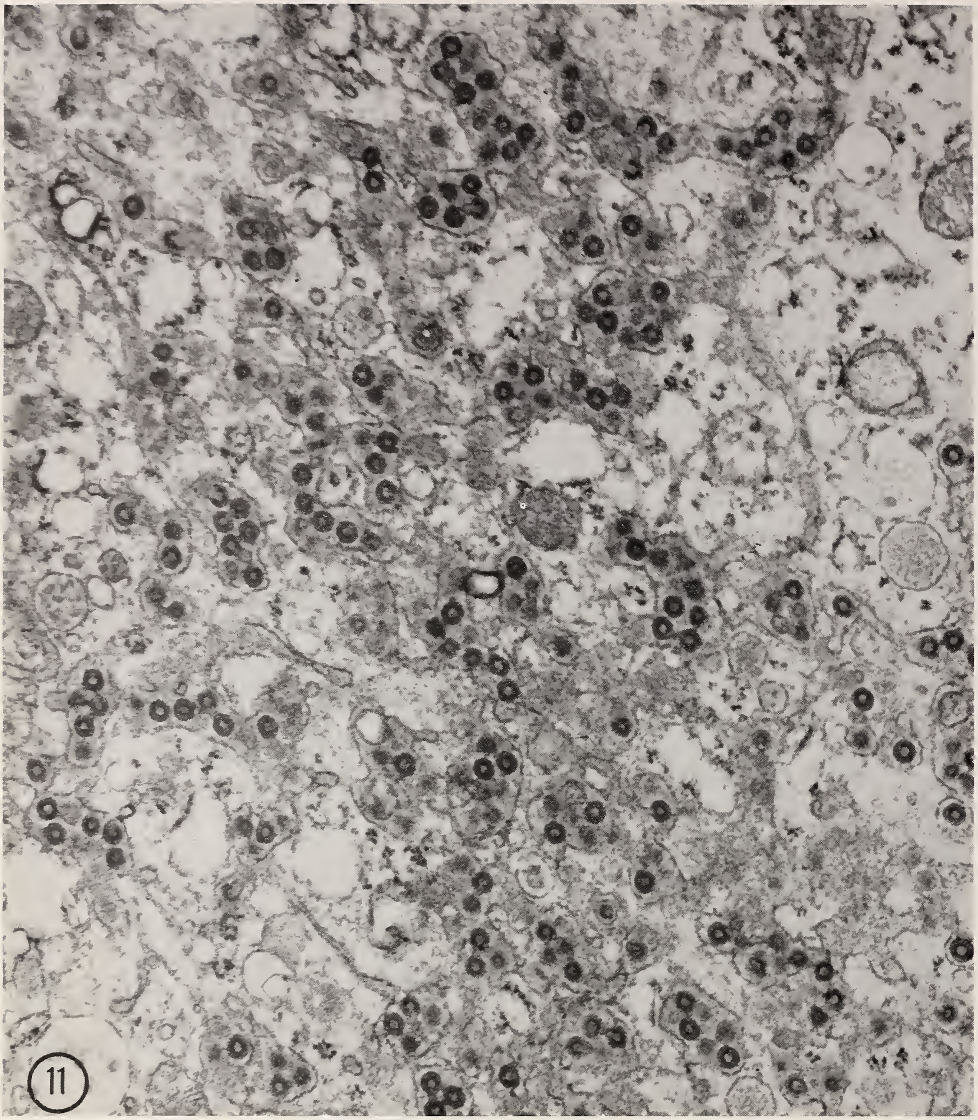
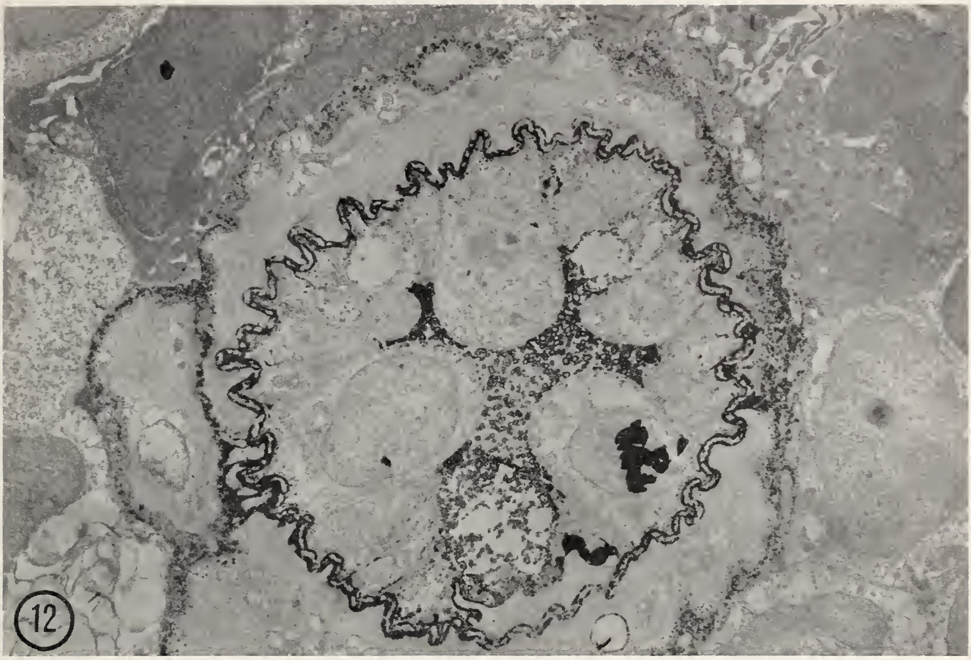


FIGURE 11.—Megakaryocyte in spleen of leukemic mice injected with MLV. After staining for alkaline phosphatase, no activity was found in the cytoplasmic channels, and associated virus particles, nor at the peripheral cell membrane. × 35,000



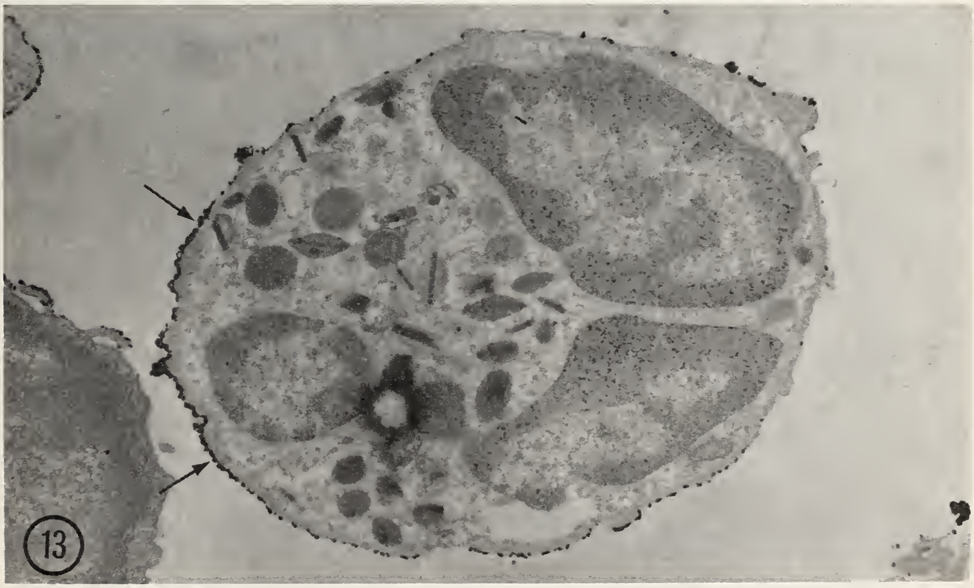


FIGURE 12.—Bone marrow of leukemic mice injected with Moloney leukemia virus. While most immature cells are negative for alkaline phosphatase, vessels show an intense activity in the subendothelial space and a weak activity at the more external membrana elastica. × 5,100

FIGURE 13.—Same tissue as that of figure 12. Mature eosinophil shows activity at the cell membrane (*arrows*). × 13,200

DISCUSSION

Dr. Metcalf: I must compliment Dr. de-Thé on his beautiful pictures. The question of the alkaline phosphatase content of some Moloney virus particles and not of others raises an interesting point. The normal thymus lymphocyte has no detectable alkaline phosphatase activity, whereas the thymic lymphoma cells do (Metcalf, D., Sparrow, N., and Wyllie, R., Aust. J. Exp. Biol. Med. Sci. 40: 215, 1962). I wonder whether it might not be worth considering that the virus may induce alkaline phosphatase activity in the lymphoma cell, whereas it might not be capable of inducing such enzyme activity in other cell types, for example, the megakaryocyte, and it is for this reason that virus emerging from lymphoma cells becomes coated with alkaline phosphatase activity, whereas virus emerging from megakaryocytes does not. This may be the origin of the heterogeneity in alkaline phosphatase content of different virus particles.

This phenomenon may be a very interesting example of a viral induction of enzyme formation in a mammalian cell.

Dr. Oroszlan: Dr. de-Thé, if I understand correctly, you assume electrostatic binding of the enzyme on the virus surface. I wonder whether you tried to remove the enzyme with some type of ionic displacement using for example strong polycationic substances such as protamine.

Dr. de-Thé: We are planning to do so, but we have not done it yet.

Dr. Lin: Can you dissociate the ATPase from the virion?

Dr. de-Thé: In the avian myeloblastosis virus system, Baluda claimed that in Tris (hydroxy methyl)aminomethane-buffered saline alone, the virus loses its adenosinetriphosphatase activity, whereas there is no loss in the presence of serum. No experiments were apparently undertaken to study the mechanism of such a loss.

Dr. Lin: Do you assume that the full viral particle penetrates into the cell?

Dr. de-Thé: After the viral particle is adsorbed at the cell surface, a pinocytotic vacuole is formed, which engulfs the particle, and only the nucleic acid of the particle apparently penetrates into the cytoplasm. What I want to suggest is that the cell-derived proteins present at the virus envelope may play a role in the adsorption and/or the penetration of the virions into the different target cells. The results showed by Dr. Graffi may support this hypothesis: The virus, isolated from different organs of mice injected with the Graffi virus, apparently induced different types of leukemia.

Dr. Salaman: I have a comment for Dr. de-Thé in regard to his suggestion that an enzyme on the surface of the particle might assist that particle to enter a cell. Surely it's difficult to believe that such an enzyme would help a particle get into a cell which already possessed the enzyme in its membrane. It is not possible that those particles having these enzymes are enabled to emerge from one type of cell, in which the bulk of their multiplication takes place, and then enter another kind of cell for the malignant transformation which they subsequently carry out?

This seems to me to support the idea, which many people have had in the past, that virus production and malignant transformation occur in different sites, or rather in different cells.

Dr. Bases: In view of what Dr. de-Thé has mentioned, it is appropriate to point out that mammalian cells are richly endowed with surface ribonuclease. I have just completed a study of this with Dr. Joseph Huppert at the Institut Gustave Roussay in Paris. That group, in addition, has found that purified avian myeloblastosis virus [Rosenbergowa, M., La Cour, F., and Huppert, J., C R Acad. Sci. (Paris) 260: 5145–5148, 1965] has always associated with it large amounts of ribonuclease. The enzyme may be of cellular origin. In any case, it could be eliminated by enzymatic digestion (Huppert, J., and Semmel, M., Biochem. Biophys. Acta 108: 501–503, 1965) and this has recently led to the isolation of high molecular weight RNA from avian myeloblastosis virus (Huppert *et al.*, Cancer Res. In press).

The point you demonstrated about the enzymes, Dr. de-Thé, is a good one. There are doubtless many enzymes on or associated with these viruses. Knowing about the ribonuclease led directly to the isolation of AMV RNA.

Using different methods, Robinson *et al.* (PNAS 54: 137–144, 1965) recently isolated 64S Rous virus RNA and I understand that high molecular weight NDV RNA also has been isolated by the same group. It appears that ribonucleases are bound to various myxoviruses and have been a source of trouble ever since the first attempts to extract infectious nucleic acids from these viruses were made. This difficulty is now being overcome by a variety of newer methods.

Isolation of the Nucleic Acid of Rauscher Murine Leukemia Virus[1]

Peter T. Mora, Vivian W. McFarland, *and* Samuel W. Luborsky, *Macromolecular Biology Section, National Cancer Institute,*[2] *Bethesda, Maryland*

We have reported previously that a low-molecular-weight (4 to 8s) RNA can be extracted from the Rauscher murine leukemia virus (*1*). The extreme liability of the RNA of this virus made it difficult to obtain a higher molecular weight material. Isolation of a high-molecular-weight nucleic acid from this virus would be desirable for several reasons. It would provide a representative animal virus RNA molecule for chemical and physical analyses: for examining, for example, the composition of this nucleic acid, whether the virus RNA occurs in a single- or in a double-stranded form, or whether one or more nucleic acid molecules are present within one virion. Of course, to be meaningful, such work should be based on an intact viral nucleic acid rather than on a degradation product. Moreover, an undegraded nucleic acid should provide the best material for testing the capability of the RNA to infect cells and cause replication of infectious virus.

Previous experiments by us (*1*) and others (*2, 3*) on related membrane viruses provided the basis for a purification and extraction procedure that provided us with an extract containing a high-molecular-weight RNA. This communication describes the isolation and the physical characterization of such an RNA from the Rauscher murine leukemia virus. This RNA exhibited a sedimentation coefficient of 73s, commensurate with a molecular weight of 13×10^6, and behaved as a single-stranded RNA in thermal denaturation experiments.

MATERIALS AND METHODS

Source of virus.—Concentrated stocks of the Rauscher murine leukemia virus were prepared by a method referred to previously (*1*), from the

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

plasma of viremic mice at Chas. Pfizer and Co., Inc., Maywood, New Jersey, under the supervision of Dr. F. J. Rauscher of the National Cancer Institute.[3] Tenfold concentrates of the virus, suspended in 0.05 M sodium citrate-citric acid buffer, *p*H 6.8, were delivered to us at −70° C in sealed glass vials. Special precautions were taken during shipment so that the contents of the vials would not thaw out but would remain approximately at solid CO_2 temperature.

Purification of the virus.—As was just pointed out, to obtain high-molecular-weight RNA, it was important to carry out the purification and extraction procedures as rapidly as possible. The density gradient purification, subsequent concentration steps, and the extraction step were devised to require only the minimum necessary time. All work was carried out without pause at approximately 4° C. Immediately on completion of the extraction, analytical ultracentrifugation was carried out on an aliquot of the RNA extract.

Density gradient fractionation.—The thawed virus suspension was layered on a previously prepared "step"-type sucrose density gradient. Aliquots of 60% sucrose (special density-gradient quality) were mixed with appropriate volumes of buffer solution (0.02 M sodium phosphate, *p*H 7, 0.18 M sodium chloride, and 10^{-3} M magnesium chloride were used for all experiments reported here) to obtain solutions with the following densities (at 30° C): 1.27, 1.20, 1.18, 1.16, 1.14, and 1.12 g per ml. The more dense solutions were carefully layered under the lighter ones in a tube of the SW 25.3 Spinco swinging-bucket rotor, using 2 ml of each solution. These gradients were overlaid with 4.9 ml of the Pfizer virus preparation. Six such tubes were then centrifuged at 22,500 rpm in the Spinco Model L-2 ultracentrifuge for 1½ hours. A yellowish, lipid-rich material remained in a thin zone at the top of the gradient. The main virus zone, used in subsequent steps, concentrated immediately above the 1.16 boundary. The buoyant density of the Rauscher virus in sucrose is known to be 1.154 g per ml at 30° C (*4*). Just above and just below this main zone two small satellite zones appeared, which were recovered separately and set aside, together with a small aliquot of the main virus zone, for electron microscopy and for sedimentation in the analytical ultracentrifuge.

The main virus zone was recovered in 24 ml, and 22.4 ml of this was diluted with 50 ml of the above-mentioned buffer solution, containing 5 μg per ml of polyglucose sulfate, sodium salt, as a ribonuclease inhibitor (*5*). This solution was centrifuged at 5° C for 120 minutes at 25,000 rpm in the Spinco SW 25.2 rotor to sediment the virus. The supernatant was aspirated and discarded, the tubes were inverted, and the walls wiped with tissue paper. Small pellets of the virus were apparent in the bottom of the tubes. The pellets were resuspended in 2 ml buffer, containing a higher concentration, 50 μg per ml of polyglucose sulfate. A sample, 0.13 ml, of the resuspended virus was set aside for various analytical determinations, which will be reported in a separate publication (*6*).

---

[3] We thank Dr. F. J. Rauscher for the virus samples.

Extraction of the RNA.—The rest of this virus suspension was adjusted to 3.5 ml with buffer solution containing 50 μg per ml polyglucose sulfate and brought to a final concentration of 1% sodium lauryl sulfate. The suspension was then triturated with a glass rod for 15 minutes to break up any remaining aggregates.

An equal volume of phenol, saturated previously with the buffer solution, was added and the mixture was shaken vigorously by hand for 15 minutes. For the separation of the phenol and the aqueous layers, the tube was centrifuged in the Servall centrifuge for 15 minutes at 10,000 × *g*.

Glass tubes were used and stoppers were covered with aluminum foil to minimize the chance of phenol-extractable material contaminating the RNA extract. The aqueous phase was pipetted off and similarly reextracted 2 more times with equal volumes of phenol. The final aqueous phase was extracted 6 times with ether to remove phenol and was then bubbled with nitrogen to expel the ether. Finally, 1.3 ml of aqueous solution was obtained, which showed a characteristic nucleic acid ultraviolet absorption spectrum with a peak at 262 mμ, representing 1.18 optical density units. An aliquot of this "RNA extract" was then diluted and used immediately for a sedimentation velocity experiment in the analytical ultracentrifuge. The rest of the extract was stored in the refrigerator.

The next morning this major portion of the RNA extract, which had been continuously refrigerated, was divided into two portions. One portion was used for another sedimentation velocity experiment (about 16 hr after extraction). Simultaneously, the other portion was used for the thermal "denaturation" experiment described below. Thus, we sought to assure the molecular integrity of the material subjected to the thermal denaturation measurements.

Analytical ultracentrifugation.—All analytical ultracentrifugations were performed at room temperature in the Spinco Model E Analytical Ultracentrifuge equipped with ultraviolet absorption optics. The solvent was the 0.2 M buffer solution, containing 50 μg per ml polyglucose sulfate, for all experiments reported. This substance has no detectable ultraviolet absorbancy at the concentration used. The "intact virus," recovered from approximately isodense sucrose, was dialyzed against the buffer solution before ultracentrifugation. Solutions were adjusted to absorbancies at 260 mμ of 0.75 to 0.90 for velocity sedimentation.

Thermal denaturation.—Measurement of the absorbancy changes with temperature was performed in the Zeiss PMQ II spectrophotometer equipped with a thermostatic cell holder, with a calibrated thermistor immersed in a cuvette. Temperature was controlled and measured with an apparatus built in our laboratory [4] according to the design outlined by Felsenfeld and Sandeen (*7*). Absorbancy readings at every temperature were corrected for volume expansion of water and for scattering. The scattering correction was performed as suggested by Leach and Scheraga (*8*), from absorbancy readings in the nonabsorbing region, 310 to 350 mμ.

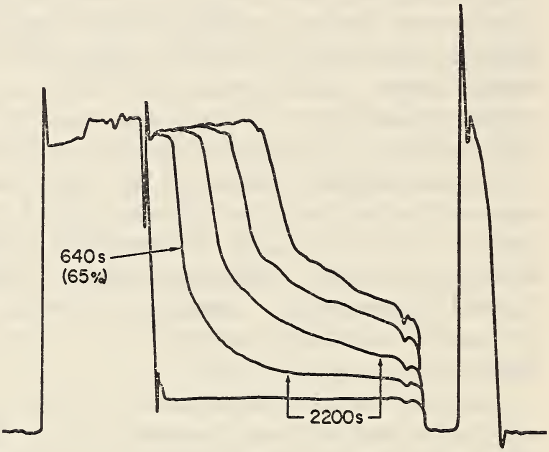
---

[4] We thank Dr. Carl Smith, who built the equipment, for help with this experiment.

RESULTS

Physical Characterization of the Density Gradient Purified Virus

Electron microscopy of the virus recovered from the main zone showed virtually pure "C-type" virus particles (*9*), characteristic of murine leukemia virus, many of them with "tail-like" elongated appendages (figs. 1 and 2). These deformations are probably caused by osmotic changes during the dilution of the sucrose medium with the buffer solution.[5]



TEXT-FIGURE 1.—Densitometer tracings of ultraviolet absorption photographs taken during analytical ultracentrifugation of the Rauscher virus after density gradient purification (described in text). Tracings show the boundary during acceleration and at 2-minute intervals thereafter (*left to right*) at 15,220 rpm. The sharp sedimentation boundary of the slowest moving component (640s) represents about 65% of the total ultraviolet-absorbing material (*ca* 260 mμ). An upper limit (2200s) to the sedimentation (of presumed aggregates) was estimated between the *two arrows* as the slowest position still apparently in the plateau region.

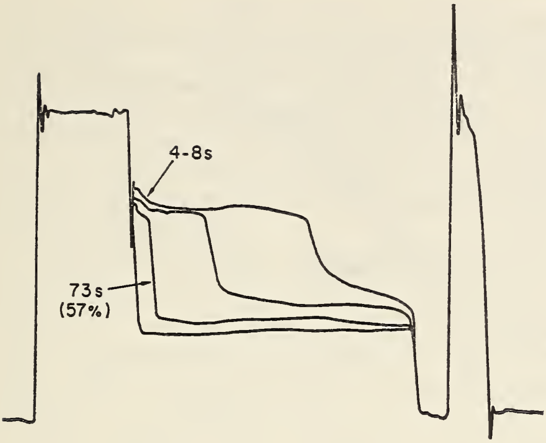
The sedimentation of the intact virus from the same zone, after dialysis against the 0.2 M buffer solution, is shown in text-figure 1. The sharp boundary of the slowest moving component sediments at a rate of 640s.

Sedimentation of the Virus RNA Extract

Immediately after completion of the extraction procedure, the analytical ultracentrifugation of the RNA extract gave the results shown in figure

---

[5] We thank Dr. Guy de-Thé for electron microscopy and for interpretation.



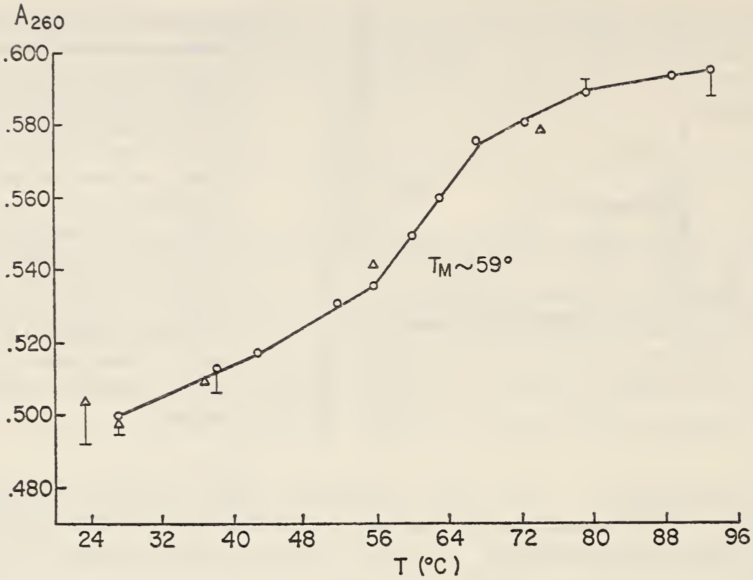
TEXT-FIGURE 2.—Densitometer tracings obtained from the ultraviolet absorption photographs of the sedimentation of Rauscher virus RNA extract shown in figure 3. Given are tracings for photographs at 1, 3, 7, and 11 minutes of centrifugation. The sharp 73s sedimenting boundary represents about 57% of the total ultraviolet absorbing material (*ca* 260 mμ). A small amount of 4 to 8s material is also present.

3 and text-figure 2. While some nonsedimentable material and a small amount of 4 to 8s material are present, there is a considerable amount of faster sedimenting material. As shown in text-figure 2, the 73s sedimenting boundary represents 57% of the ultraviolet-absorbing material. On completion of the sedimentation, the cell was refrigerated and gently shaken for about 10 hours to resuspend the sedimented material. When the same cell was rerun the following morning in the ultracentrifuge, a very diffuse boundary, representing only 30% of the total ultraviolet absorbancy, sedimented at 67s. Obviously, there was extensive degradation of the material, from 57% of 73s to 30% of only 67s size, after exposure to room temperature for about 3 hours in the ultracentrifuge cell, during the sedimentation velocity experiment.

A second sample was taken the following morning from the original RNA extract, which had been refrigerated since the extraction was completed. Ultracentrifugation indicated that there was still 50% of the original 73s material present. At the same time, another aliquot of the original extract was subjected to a thermal denaturation experiment.

Thermal Denaturation of the RNA Extract

The absorbancy changes at 260 mμ upon heating and cooling are shown in text-figure 3. The gradual hyperchromic transition and the relatively low median temperature of the transition ($T_m = 59°$) in the 0.2 M buffer solution used as solvent are similar to the behavior of known single-stranded RNA molecules, such as the tobacco mosaic virus (TMV) RNA (*10*). The melting experiments were carried out on assuredly high-molecular-weight RNA (50% of 73s material). It can be seen that the absorbancy begins to rise immediately upon heating, at relatively low temperatures. Upon cooling, part of the hyperchromicity is lost and a lower absorbancy recovered. After the heating and cooling cycle, ultra-



TEXT-FIGURE 3.—Thermal denaturation behavior of Rauscher virus RNA extract. Absorbancy at 260 mμ as a function of temperature is shown for the heating cycle (*solid line, open circles*) and for the cooling cycle (*triangles*). T_m is the median transition temperature of the hyperchromicity. Details of the experiment are given in the text. Solvent: 0.02 M sodium phosphate buffer, *p*H 7, 0.18 M sodium chloride, 0.001 M magnesium chloride.

centrifugation of this sample showed the presence of 50% of 4.4s material, and 50% nonsedimentable material.

DISCUSSION

From the melting experiment on the high-molecular-weight RNA extract in the 0.2 M buffer solution, we may surmise that this RNA molecule occurs in a single-stranded form. Especially relevant are the lower temperature points, at early times in the experiment before much degradation could have occurred. The absorbancy is seen to rise immediately. On the other hand, high-molecular-weight, double-stranded RNA, such as reovirus (*11*) or wound tumor virus RNA (*12*), exhibits essentially unchanged absorbancies until quite high temperatures are reached, before any increase is observed.

The sedimentation coefficient of the intact purified Rauscher virus (640s), together with the diameter estimated from electron microscopy (100 mμ), and the value of the buoyant density (1.16 g/ml), determined by density gradient centrifugation, allow the estimation of the viral particle weight as 2.2×10^8, assuming the particles are spherical. Although the electron micrographs (figs. 1 and 2) indicate the particles possess a fair degree of anisotropy, this was probably produced in these samples by the osmotic changes attendant upon diluting out the isodense sucrose with buffer solution. In the sample used for ultracentrifugation, the sucrose was removed more gradually, by dialysis. Therefore, the particles seen in the ultracentrifuge may be somewhat less asymmetric than the particles seen in the electron micrographs. In any case, since anisotropy would in general reduce s values, the viral particle weight estimated here may only be a minimum value.

The molecular weight of the RNA can be estimated from the sedimentation coefficient of 73s to be 13×10^6, using the empirical equation found by Spirin for single-stranded TMV RNA in 0.1 M salt (*13*). This molecular weight estimate is probably an upper limit, since our experiments were done at a concentration of 0.2 M salt, which contained 10^{-3} M $MgCl_2$. Under the conditions used (*13*) to relate s to molecular weight (0.1 M ionic strength, 0.01 M EDTA), s values should be lower for a single-stranded RNA molecule (*10*). Thus, the above-mentioned reservations concerning the estimated molecular weights of the virus particle and of the RNA extract suggest that the RNA constitutes less than 6% of the weight of the virus.

Chemical analyses of the RNA extract are in progress. We also plan to test the infectivity of such high-molecular-weight RNA extracts.

SUMMARY

A virus preparation from the plasma of viremic mice was purified by sucrose density gradient centrifugation. The sharp band containing the virus was isolated and shown by electron microscopy to contain essentially "pure" virions. The sedimentation coefficient of the purified virus was determined in the analytical ultracentrifuge equipped with ultraviolet absorption optics and found to be 640s. The virus particle weight is therefore estimated to be 2.2×10^8.

Extraction of the nucleic acid by the cold phenol method after treatment with sodium lauryl sulfate resulted in a solution with a characteristic nucleic acid spectrum. The sedimentation coefficient of this RNA, determined as indicated above, was 73s. Great sensitivity to breakdown to smaller fragments was observed. The dependence of the ultraviolet absorbancy upon temperature (thermal "denaturation") indicated that the high-molecular-weight RNA was single stranded. The molecular weight of the RNA was estimated to be 13×10^6.

REFERENCES

(*1*) MORA, P. T., and MCFARLAND, V. W.: The nucleic acid of a murine leukemia virus. Proc Nat Acad Sci USA 54: 756–763, 1965.

(*2*) ROBINSON, W. S., PITKANEN, A., and RUBIN, H.: The nucleic acid of the Bryan strain of Rous sarcoma virus: Purification of the virus and isolation of the nucleic acid. Proc Nat Acad Sci USA 54: 137–144, 1965.

(*3*) DUESBERG, P. H., and ROBINSON, W. S.: Isolation of the nucleic acid of Newcastle disease virus (NDV). Proc Nat Acad Sci USA 54: 794–800, 1965.

(*4*) O'CONNOR, T. E., RAUSCHER, F. J., and ZEIGEL, R. F.: Density gradient centrifugation of a murine leukemia virus. Science 144: 1144–1147, 1964.

(*5*) MORA, P. T.: Relative importance of electrostatic forces in the interaction of ribonuclease and ribonucleic acid. J Biol Chem 237: 3210–3214, 1962.

(*6*) MORA, P. T., MCFARLAND, V. W., and LUBORSKY, S. W.: Nucleic acid of the Rauscher mouse leukemia virus. Proc Nat Acad Sci USA 55: 438–445, 1966.

(*7*) FELSENFELD, G., and SANDEEN, G.: The dispersion of the hyperchromic effect in thermally induced transitions of nucleic acids. J Molec Biol 5: 587–610, 1962.

(*8*) LEACH, S. J., and SCHERAGA, H. A.: Effect of light scattering on ultraviolet difference spectra. J Amer Chem Soc 82: 4790–4792, 1960.

(*9*) BERNHARD, W.: The detection and study of tumor viruses with the electron microscope. Cancer Res 20: 712–727, 1960.

(*10*) BOEDTKER, H.: Configurational properties of tobacco mosaic virus ribonucleic acid. J Molec Biol 2: 171–188, 1960.

(*11*) GOMATOS, P. J., and TAMM, I.: The secondary structure of reovirus RNA. Proc Nat Acad Sci USA 49: 707–714, 1963.

(*12*) ———: Animal and plant viruses with double-helical RNA. Proc Nat Acad Sci USA 50: 878–885, 1963.

(*13*) SPIRIN, A. S.: Some problems concerning the macromolecular structure of ribonucleic acid. *In* Progress in Nucleic Acid Research, vol. 1 (Davidson, J. N., and Cohn, W. E., eds.). New York, Academic Press Inc., 1963, pp 301–345.

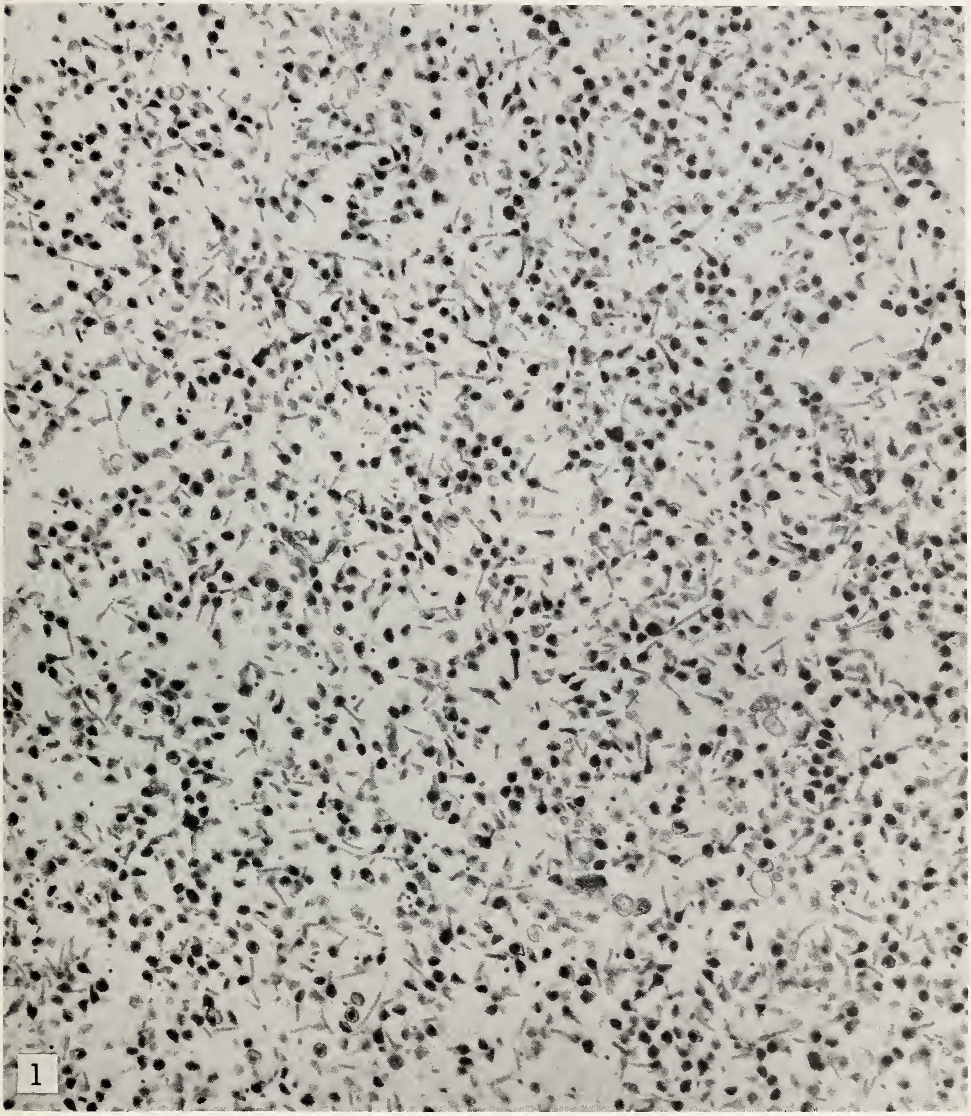


Figure 1.—Electron micrograph of thin section of Rauscher plasma virus pellet obtained from the main virus band after sucrose density gradient centrifugation (described in text). *Note* homogeneity of preparation. Most virus particles are "tailed." This is probably due to osmotic effects upon diluting the isodense sucrose solution with buffer solution to permit formation of the virus pellet during centrifugation. × 10,500

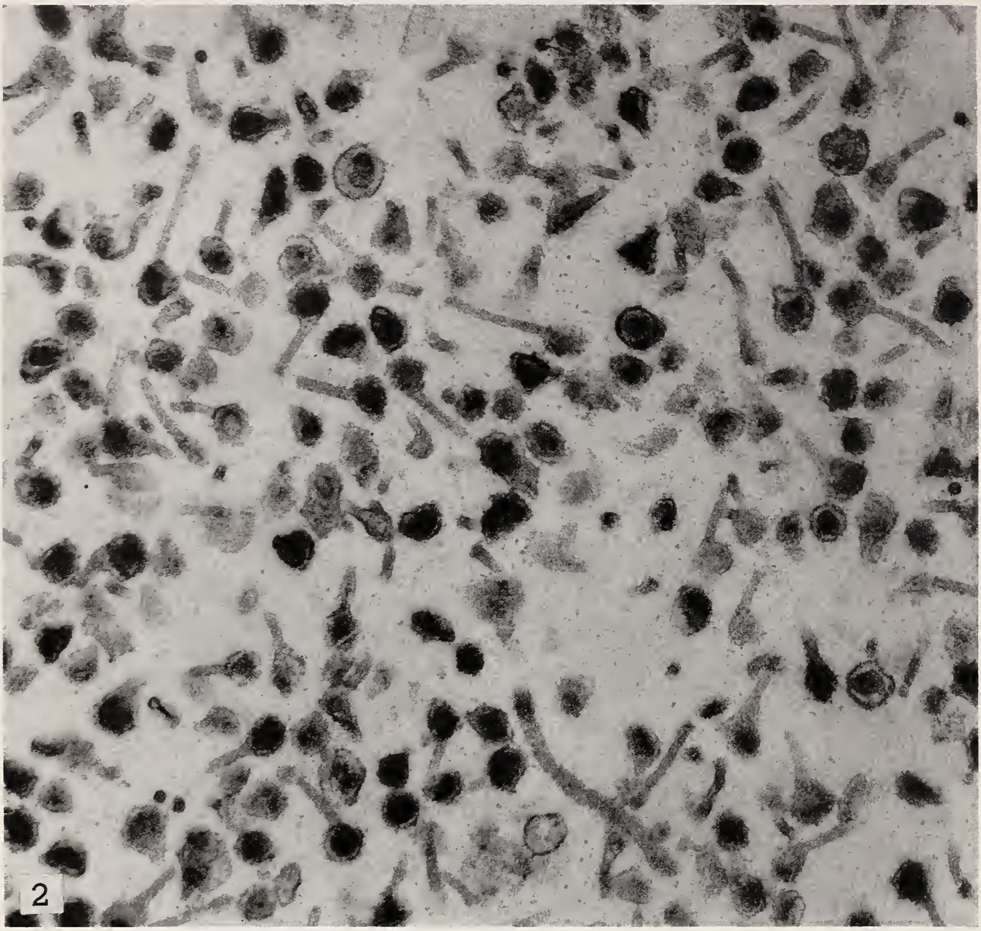


FIGURE 2.—Same as figure 1. Tail-like appendages are well visualized but no cell debris is visible. × 60,000

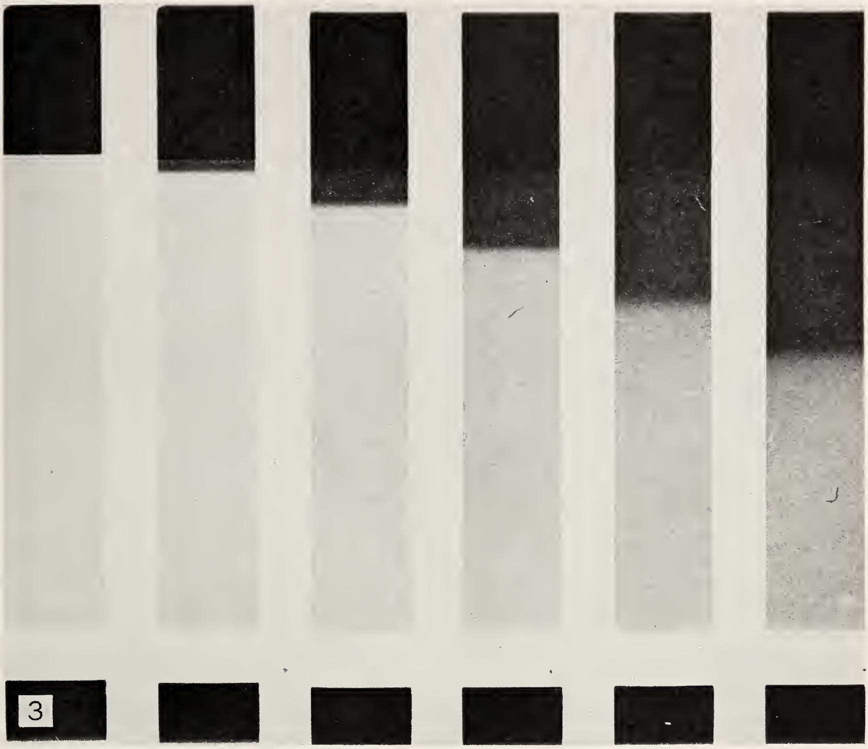


Figure 3.—Photographs taken during analytical ultracentrifugation of Rauscher virus RNA extract, immediately after extraction. Details given in text. Pictures (*left to right*) represent cell during acceleration and at 2-minute intervals thereafter, at 44,770 rpm. A single sharp sedimenting boundary is evident.

DISCUSSION

Dr. Oroszlan: I think the results Dr. Mora described represent the first case in which one was successful in obtaining RNA from a mouse leukemia virus in amounts sufficient for chemical and physical studies. It is important that the RNA isolated from the Rauscher virus appears to be the native RNA.

I have a few questions to ask Dr. Mora. First, what were the criteria besides electron microscopy for the purity of the virus from which you isolated RNA? Second, is this RNA infectious? And third, is it homogeneous or not?

Dr. Mora: As to the first question about the purity of the virus, it is very difficult to say anything more, except that the particles as shown by electron microscopy are structurally similar (*see* figs. 1 and 2 in our paper). The band at 1.16 density was free of lipid, which remained on the top of the gradient as a thin layer. The 1.16 zone contains only isodense particles with the characteristic appearance of "C" type virus particles.

As to the question about the homogeneity of the RNA, the sedimentation diagram (text-fig. 2) shows that the sharp boundary which sedimented at 73s represented 57% of the total sedimentable material. The rest of it was a small amount of 4 to 8s material which is probably degradation product, and a considerable amount—about 30% or so—of faster sedimenting heterodisperse material, possibly aggregate.

The spectrum of the whole extract in the ultraviolet was characteristic of RNA [cf. (*1*)]. The absorbancy ratio at 280/260 mμ was below 0.5, indicating sufficient purity from proteins. Thus, we assume that the virus RNA is the high molecular weight 73s material which sediments as a sharp boundary, represents the 57 to 60% of the total RNA, and corresponds to 13 million molecular weight, calculated as single-stranded RNA.

As to your question about the infectivity, we have not yet carried out the experiments. We plan to do this with Dr. Rauscher. The main point is that for the first time we have the material to do a clear-cut experiment: We have assuredly pure RNA, and enough of it as the intact viral genome so that we wouldn't have to worry that only part of the RNA sequence is present when infecting mouse cells.

Dr. Pledger: I have two questions for Dr. Mora. First, concerning the melting curves of RNA extracted from Rauscher virus, what was the molarity of the salt solution used? This can certainly affect the melting curve profile.

Second, what conclusion does Dr. Mora have as to the structure of the RNA in the intact viral particle as opposed to the RNA in solution?

Dr. Mora: The salt concentration was approximately 0.2 M. We used a 0.02 M phosphate buffer, *p*H 7, with added 0.18 M sodium chloride, and 10^{-3} M magnesium chloride. Naturally, upon heating at different ionic strengths, the transition (melting) of ultraviolet spectra is different. At the salt concentration we employed, the melting curve of an assuredly single-stranded RNA, such as tobacco mosaic virus RNA, is entirely similar to that in text-figure 3.

As far as detailed analysis of the melting curves in the whole ultraviolet region, we have the data which Dr. G. Felsenfeld is going to feed through his computerized program (Felsenfeld, G., and Hirschman, S. Z., J. Molec. Biol. 13: 407, 1965). Also, we are doing other computerized analyses together with Dr. Pratt, based on the spectra at various *p*H values (Pratt, A. W., Toal, J. N., Rushizky, G. W., and Sober, H. A., Biochemistry 3: 831, 1964).

As to your question about what is the state of the RNA in the virus versus that in the extract, please consider that the 13 million molecular weight RNA must represent the intact viral genome. Now the melting experiments show that in solution it is obviously in the single-stranded form. I do not think that during the extraction there would be a chance that we have a transition from a double-stranded RNA to single-stranded form, because of the molarity of the solutions and the low temperatures employed. With such molarity one would have to go up about 80° C, or higher, to separate a double-stranded RNA.

I think the RNA exists essentially the same way in the virus as the main component in the extract; it is a single-stranded 13 million molecular weight RNA.

I was just handed a photocopy of a recent French publication (Galibert, F., Bernard, C., Chenaille, P., and Boiron, M., C. R. Acad. Sci. 261: 1771, 1965). Essentially they observed the presence of a small amount of radioactive high molecular weight RNA of 70s, as measured by sedimentation in a sucrose density gradient of radioactive-labeled material obtained after infecting tissue cultures with the Rauscher virus in the presence of ^{32}P. The difference is that we actually isolated the RNA from purified, highly infectious plasma virus in the intact form and in good yield. But I am happy to note that the molecular weights jibe.

Dr. Sykes: I would like to bring before this august gathering an unusual observation made by Drs. A. G. and R. A. Liebelt of Baylor University and ourselves. This, briefly, is the association of a leukemia which appears to be a monocytic leukemia with a methylcholanthrene-induced transplantable hepatoma. When the hepatoma regresses spontaneously after having attained the size of 1 cm or more and is removed surgically, the animals develop a leukemia.

These hepatoma-transplant-bearing animals exhibited a characteristic hepatosplenomegaly associated with leukemia and similar to that encountered with Friend and Rauscher disease.

Dr. Gabriel Seman of Paris, who examined the blood smears, considered that these mice had a monocytic type of leukemia.

Electron microscopy of the hepatoma transplants and of the leukemia spleens demonstrated characteristic budding virus particles whose number increased with increasing transplant generations. These particles increased in numbers during a successive passage of cells derived from hepatoma and spleen in tissue culture.

This tumor was induced by feeding methylcholanthrene to one CBA mouse; thereafter it was transplanted without the use of methylcholanthrene. The normal occurrence of reticulum cell tumors and of other tumors in these mice was 3 in 500 during a 5-year period.

Murine Leukemia Viruses: Rupture With Ether and Detergents to Subviral Constituents[1]

Timothy E. O'Connor, Frank J. Rauscher, Guy de-Thé, Mary A. Fink, *and* Paul Gerber,[2] *Laboratory of Viral Oncology, National Cancer Institute,*[3] *and Division of Biologics Standards,*[3] *Bethesda, Maryland*

Fifteen years after the pioneering study of Gross, the practicing virologist has available for his study a range of murine leukemia viral isolates associated with the names of many of the distinguished participants of this Conference. Recent immunological studies have explored the possible interrelationship both among some of these agents and the tumors they induce. Some antigenic relationships and dissimilarities have been established for a few of these viruses (*1–3*), but the structural entities corresponding to the shared or dissimilar antigens involved are presently unknown. Electron microscopic studies show that the murine leukemia virions, like other membrane-enclosed viruses, are complex, multicomponent structures assembled at the membrane of the infected cell. Accordingly, it would appear desirable to explore the disassembly of the virions of their subviral structural units to determine the structural basis of the various antigens. Furthermore, elucidation of the morphology of the viral subunits should aid in the physical classification of the murine leukemia viruses as a group *vis-à-vis* other groups of membrane-enclosed viruses.

It may be instructive to consider the advantages resulting from this type of approach in the study of myxoviruses. The effects of ether treatment of myxovirus have been reviewed by Schafer (*4*). Briefly, when myxoviruses are treated with ether, the lipoprotein-rich viral membranes rupture and the inner viral nucleoproteins are exposed. Hemagglutinins, associated with the viral membrane, can be removed by adsorption to erythrocytes, and preparations of the viral nucleoprotein, which can be further purified by cycles of low and high speed centrifugation, are thus obtained. The hemagglutinins can then be eluted from the erythrocytes by appropriate incubation procedures. Purified nucleoprotein preparations of different strains of the same virus generally behave as group antigens (S antigens) and show extensive serological cross reactions but little cross reactions with the viral-specific antigens (V antigens) associated with the viral membrane. Electron microscopic examination of ether-damaged myxoviruses (*5*) or the purified viral fission products (*6*) revealed that the inner components consisted of rod or ropelike structures, frequently with a hollow inner core and helical symmetry. The inner structures derived from Newcastle disease virus (NDV), Sendai, and mumps (*5*) show a general morphological resemblance to some of the rod-shaped plant viruses, whereas those derived from influenza virus appear to consist of a more loosely packed helical structure (*6*) with a smaller diameter.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Division of Biologics Standards.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Recently Eckert *et al.* (*7*, *8*) observed a quite different pattern of rupture on treating avian myeloblastosis virus (AMV) with ether together with the detergent Tween 80. They detected no large viral fragments. Instead, an antigenically active fraction having both a low density (1.06) and sedimentation coefficient (2.3s) was found. Sera of rabbits, immunized with this fraction, specifically fixed complement on exposure to density gradient purified AMV. Electron microscope examination revealed no definite organized structures in the viral fission product.

These studies on myxoviruses and AMV indicate that the Tween-ether rupture is a useful tool which 1) can aid in the classification of membrane-enclosed viruses—the classification can be guided both by the over-all pattern of rupture as well as by the dimensions and morphology of uncovered nucleoproteins; 2) can aid immunological studies in that the uncovered nucleoproteins obtained from different strains of a virus frequently show extensive serological cross reactions while showing little cross reaction with the exterior viral antigens.

We shall present some results from our ongoing studies on the ether-detergent rupture of the Rauscher and Moloney murine leukemia viruses. We have previously reported on the comparative rupture of the Rauscher virus, influenza virus (PR8 strain), and NDV on treatment with ether as shown by density gradient centrifugation (*9*). A more detailed account of the structures of the products obtained on ether-rupture of Rauscher virus (*10*) is presented elsewhere.

MATERIALS AND METHODS

Viruses.—Stocks of the Rauscher and Moloney viruses, each suspended in 0.05 M sodium citrate buffer, *p*H 6.75, were prepared as tenfold purified concentrates of the plasma of viremic BALB/c mice by the methods of Rauscher (*11*) and Moloney (*12*). Rauscher virus obtained from culture fluids of an infected monolayer mouse-cell culture (JLSV5) and Moloney virus obtained from culture fluids of an infected mouse-cell suspension culture (MT 77) were concentrated and purified by centrifugation on potassium tartrate gradients in the Anderson continuous-flow type-B zonal ultracentrifuge rotor. The 1.16 density zones, containing the viruses, were dialyzed against 0.05 M sodium citrate. These various virus stocks were prepared at the John L. Smith Cancer Memorial Laboratory of the Chas. Pfizer Co., Maywood, New Jersey, under contract to the National Cancer Institute. Virus stocks were frozen at −70° C in sealed ampoules until used. The purification of the viruses from tissue culture fluids was performed by Mr. J. Toplin.

Tween-ether treatment of viruses.—The various virus suspensions were thawed, and sufficient 0.7 M sodium citrate solution, *p*H 6.75, was added with stirring to adjust the molarity of the citrate suspension medium to 0.153 M. After standing for 30 minutes at 4° C, each virus suspension was clarified by centrifugation at 5,000 × *g* for 10 minutes. A very small pellet generally was obtained. The clarified viral suspensions were then centrifuged (5 ml portions) in Lusteroid tubes on the SW39L Spinco rotor for 1 hour at 22,500 rpm to pellet the virus. Dulbecco's phosphate-buffered saline (PBS), *p*H 7.4, was added to the viral pellets which, after standing for 1 hour, were resuspended by gentle pipetting. The final concentration of Rauscher virus was about 50× that of the viral concentration in the plasma of viremic BALB/c mouse plasma. The Moloney virus concentrates, prepared either from tissue culture or plasma, were adjusted to an equivalent concentration, as judged by ultraviolet absorbance.

Tween 80 (polyethylene oxide-sorbitan-monooleate, supplied by Hilltop Laboratories, Cincinnati, Ohio) dispersed in PBS (8 mg/ml) was added to the viral suspensions to a final concentration of 2 mg per ml. The Tween-viral suspensions were thoroughly mixed on a Cyclo Mixer (Clay Adams Inc., New York, N.Y.) operating at top speed for 30 minutes at 4° C. Ether (one-half volume of Merck anesthesia grade) was then added and the mixture was alternated between an ice bath and the Cyclo Mixer operating at full speed for 1 hour. In some experiments, benzene (one-half volume) was then added and mixing was continued for another hour. The resulting emulsion was then spun at low speed (1000 × *g*) on a Clinical Centrifuge (International Equipment Co., Boston, Mass.) with a lower aqueous phase, an upper ether phase, and a gelatinous interphase resulting. The aqueous phase was removed with a Pasteur pipette. The ether phase and interphase were then twice washed with a half volume of PBS—5-minute mixing on the Cyclo Mixer followed by centrifugation to separate phases—and the aqueous washings were added to the aqueous phase. The resulting aqueous phase and washings were then washed twice with one-quarter volume portions of ether, and the last traces of ether were removed from the washed aqueous phase by a stream of nitrogen blown over the surface for several hours. The resulting solution will be referred to as the "aqueous extract." The washed ether phase and accompanying interface gel were then mixed with an equal volume of PBS and the ether removed in a stream of nitrogen. The resulting cloudy suspension will be referred to as the "ether extract." In some experiments both the aqueous extract and ether extracts were separately mixed on the Cyclo Mixer with half volume of Freon 113 (E. I. duPont and Co., Wilmington, Del.).

Sodium deoxycholate-ether treatment of viruses.—The procedure was identical to that of the Tween-ether treatment except that sodium deoxycholate (Fisher Scientific Co., Chicago, Ill.), at a final concentration of 2 mg per ml was used in place of the Tween 80.

Centrifugation of the aqueous extracts and ether extracts.—The aqueous extracts were centrifuged at 5000 × *g* for 10 minutes. No detectable pellet formed. After the centrifugation was continued for 3 hours at 22,500 rpm on the SW39L Spinco rotor, the blue opalescence originally present in the solutions disappeared and appreciable pellets (P1) resulted. The pellets (P1) were digested and resuspended in 0.5 ml portions of 1% ammonium acetate solution in water. The resuspended P1 materials were clarified by low speed centrifugation (5000 × *g* × 5 min) and then centrifuged in 0.8 ml cellulose tubes (Beckman tube #305528) in the Spinco SW39L rotor, with the use of swinging buckets equipped with insert adapters (Beckman adapter 305527), at 22,500 rpm for 3 hours. The resulting pellets were designated P2.

The ether extracts were centrifuged at 5000 × *g* for 10 minutes and a considerable pellet resulted. Further centrifugation of the supernatant at 22,500 rpm on the SW39L Spinco rotor for 1 hour completely removed the residual opalescence and gave a further pellet.

Density gradient centrifugation.—The virus stocks after clarification in 0.153 M sodium citrate, the virus stocks after admixture with Tween 80 in PBS, the aqueous extracts and the ether extracts obtained in both the Tween-ether treatment, and the pellets obtained in the centrifugation of the products of the Tween-ether treatment were all examined by density gradient centrifugation. The centrifugation on preformed gradients of potassium citrate or sucrose was performed according to the previously published method (*13*). Generally, 0.5 ml samples were centrifuged on 4.5 ml potassium citrate gradients (density 1.4–1.0 measured at 30° C) for 2 hours, or on sucrose gradients (density 1.30–1.0 at 30° C) for 3 hours at 4° C on the SW39L rotor at 35,000 rpm. Three ml samples were centrifuged for 4 hours on potassium citrate or sucrose gradients (25 ml) on the SW25-1 rotor at 25,000 rpm. Bands forming on the gradients were removed by lateral puncture of the tubes at the band site and extraction of the contents of the bands with a hypodermic syringe having a 27-gauge needle.

Electron microscopy.—Negatively stained preparations were made with 2% phosphotungstic (PTA) adjusted to *p*H 7 to which a half volume of 0.08% bovine serum albumin in water was added. The stained preparations were examined with a Siemens Elmiskop 1A electron microscope on nickel grids having Formvar carbon-coated membranes. Occasional samples were fixed with osmium tetroxide vapors, but since both fixed and unfixed Tween-ether-treated Rauscher virus preparations showed no differences in structure, most of the Tween-ether preparations were studied with no fixation. The pellets P1 and P2 obtained in the Tween-ether treatment of the viruses were resuspended in a minimal quantity of 1% ammonium acetate in water solution, and these solutions were then clarified and examined by negative staining.

For embedding, pellets were fixed for 2 hours with 2.5% glutaraldehyde buffered at *p*H 7.4 with sodium cacodylate (*14*). The pellets were rinsed for 30 to 60 minutes in cacodylate buffer, *p*H 7.2, and they were then dehydrated and embedded in Epon araldite (*15*). Thin sections were double stained with uranyl actetate (*16*) and lead citrate (*17*) and examined at the same magnification as that of the negatively stained preparations.

Complement fixation.—The quantitative complement fixation (CF) test of Wasserman and Levine (*18*) as modified by Gerber and Birch (*19*) was used. The virus antigen preparations, as isolated from sucrose density gradients, were extensively dialyzed against Veronal buffer (*20*). Sera were collected from Osborne-Mendel rats which carried a transplantable Rauscher virus-induced tumor at the time of maximum tumor development (21–30 days post transplantation) and from BALB/c mice immunized with formalin-inactivated Rauscher virus (*21*). All sera were heat-inactivated for 20 minutes at 60° C. The degree of CF was expressed as the percent ratio of the released hemoglobin as measured by the optical density at 413 mμ in the presence of the antigen-antibody mixtures as compared to the antibody controls.

Immunodiffusion.—The experimental procedures used are described by Dr. Fink in her paper at this Symposium.

RESULTS

Both the Rauscher and Moloney viruses—derived either from viremic mouse plasmas or tissue culture fluids—behaved quite similarly on treatment with ether and detergents. Accordingly, the effects of the ether-detergent treatment on Rauscher plasma-derived virus will be described in detail as representative of those observed with the various stocks of both viruses.

Stained, thin sections of pellets prepared from the Rauscher plasma-derived virus, after a preliminary low speed clarification of the virus suspensions in 0.153 M sodium citrate, showed a quite homogenous, clean, viral preparation (fig. 1).

On density gradient centrifugation of the virus through either potassium citrate (*13*) or sucrose gradients (fig. 2), a single opalescent band was obtained which contained the viral infectivity. When the material in the band (after extensive dialysis against PBS) was treated with formalin and injected, together with Freund's adjuvant, into BALB/c mice (*21*), it conferred complete immunity to the Rauscher disease on subsequent challenge with live Rauscher virus. In a micro Ouchterlony immunodiffusion experiment, the band isolate from the sucrose gradient (without further dialysis) gave specific precipitation when reacted with antisera prepared against the Rauscher virus in the BALB/c mouse, guinea pig, or monkey. Details of this type of experiment will be reported by Dr. Fink.

The virus band isolate obtained from the sucrose gradient, after extensive dialysis against Veronal buffer, fixed complement in the presence of appropriate antisera (table 1). Immune sera from BALB/c mice previously immunized with virus (col 1), or from rats bearing a transplantable Rauscher virus-induced tumor (col 3) gave high fixation. Interestingly, the band isolate appreciably bound complement in the presence of antiserum to normal BALB/c mouse antigens (col 5). This may indicate the presence of normal mouse antigens either adsorbed to, or structurally integrated into, the density gradient purified virions.

The results obtained in these density gradient centrifugation experiments indicate that the various biological properties of the virus are associated with a common density.

After treatment with Tween 80 alone (1.5–10 mg Tween/ml virus in PBS) the Rauscher virus still yielded a single 1.16 density band on density gradient centrifugation. A quite different result was obtained when the products of the Tween-ether treatment of the virus were examined. The behavior of the ether extract (left tube) and of the aqueous extract (right tube) (*see* Materials and Methods) on density gradient centrifugation in identical potassium citrate gardients is shown in figure 3, which illustrates the results obtained in an early experiment. The 1.16 density opalescent band containing intact virus (faint band near middle of the right tube) was almost completely eliminated and aqueous extract yielded a denser band (density 1.24–1.27), whereas the ether extract contained very low density opalescent materials. This particular experiment was somewhat atypical in that a very high concentration of virus (100× plasma concentration) was used and this appeared to exert a slight protection for the virus in the Tween-ether treatment. In subsequent ex-

TABLE 1.—Complement-fixing activity of purified murine leukemia virus (Rauscher)

Antigens	Rauscher BALB/c	Normal BALB/c	Sera, Rauscher tumor, rat	Normal rat	Anti-normal BALB/c plasma, guinea pig
Rauscher BALB/c plasma sucrose band	98*	0	96	0	45
Normal BALB/c plasma	0	0	0	0	98

*Percent fixation of complement.

periments using somewhat lower concentrations of virus (10–50× plasma concentration) no normal virus was detected after the Tween-ether treatment, and use of the washing procedure described in Materials and Methods completely removed any typical ether-extract material present (opalescent band near surface of right tube) as a contaminant in the aqueous extract.

The role of the Tween detergent appeared to be promotion of efficient emulsification of the ether and aqueous phases and thus interaction of the ether with the virions.

Use of sodium deoxycholate (2 mg/ml) as the detergent gave essentially equivalent results, and, as shown in figure 4, the aqueous extract obtained consisted of material with a density (1.24–1.27) higher than that of the untreated virus. Treatment with sodium dodecyl sulfate and ether also caused apparent dearrangement of the virus, but a more complex range of products was revealed by density gradient centrifugation. Intact virus and a range of denser components (1.16–1.23) were detected in the aqueous extract after treatment of the virus with ether alone and was interpreted as indicating an incomplete and variable dearrangement of the virions.

In several experiments, mixtures of benzene and ether together with Tween were used in the treatment of virus, with, however, no significant change in the nature of the resulting aqueous extract as compared to the Tween-ether product, judged by density gradient centrifugation. The 1.24 to 1.27 density material of the aqueous extract was not removed from PBS suspension on high speed mixing with the chlorofluorocarbon Freon 113. In contrast, the intact Rauscher virus was removed from PBS suspensions—judged by disappearance of the typical 1.16 density opalescent band on density gradient centrifugation—by a few cycles of similar high speed mixing with the Freon 113. The opalescent components in the ether extract were also largely removed from suspensions in PBS when they were mixed with Freon.

The ether extract appeared to contain much aggregated material which sedimented as a quite large pellet when the extract was centrifuged at 10,000 × *g* for 5 minutes. The remaining opalescence was essentially removed on centrifugation for 30 minutes at 30,000 × *g*. When the ether extract was centrifuged on very shallow potassium citrate gradients, it appeared resolvable into several low density components, which are being further characterized. The aqueous extract appeared to consist mainly of material with a sedimentation behavior comparable to that of the intact virus: Little of the opalescent material was sedimented on centrifugation at 10,000 × *g* for 5 minutes, most at 30,000 × *g* for 30 minutes, and practically all at 80,000 × *g* for 3 hours.

The low density material in the ether extract, when isolated from a sucrose density gradient, contained viral antigen and in the micro Ouchterlony immunodiffusion test specifically reacted with antisera prepared against the intact virus. On the other hand, the 1.24 to 1.27 density material isolated from sucrose density gradients of the aqueous extract have so far failed to react in the micro Ouchterlony test with immune sera prepared against the intact virus.

Electron microscopic examination of the negatively stained preparations of the ether extracts gave an impression of fragmented, debris-like material, but the technical quality of the preparations was not sufficient to permit conclusions as to the morphology of the constituents.

Thin sections of pellets (P1 and P2) from the aqueous extract did not show any intact virions. Instead, two types of particles, each devoid of the outer membrane, which is a constituent of the intact virus, were detected. The predominant particles were 650 to 700 A in diameter, and although they were generally spherical they often had angular profiles. After being stained with uranyl and lead salts, they had a dense periphery, whereas their centers had a lower electron density and apparently contained fibrillar material. The second and less frequently observed type of particle was roughly comparable in size but had the general appearance of a doughnut with some amorphous material attached to its periphery.

Negatively stained preparations of resuspended pellets P1 and P2 of the aqueous extract also showed two types of particulate structures. One of these (fig. 5) appeared as spherical bodies 700 to 750 A in diameter with dense peripheries enclosing fibrillar material. The most prominent feature of the second type of structure (fig. 6) was a ringlike profile. This circular profile had an over-all diameter of 750 to 850 A, was 70 to 75 A thick and had a hollow, 15- to 25-A wide, electron-lucent core. Some portions of the profile gave an impression of periodicity along both the outer and inner circumferences. The morphology of these particulate structures observed on electron microscopy was not altered by Freon treatment of the aqueous extract.

DISCUSSION

When either the Rauscher or Moloney virus was treated with ether, together with one of the detergents Tween 80 or sodium deoxycholate, the viruses of normal 1.16 density were eliminated and both lower density (ether extract) and higher density (aqueous extract) products were formed.

This observation, *per se*, does not permit an unequivocal interpretation. The possibility that the ether-detergent treatment had removed a low density, adsorbed impurity from the virions has to be considered. In this interpretation the 1.24 to 1.27 density material in the aqueous extract would represent native virus and the low density ether extract, the stripped impurity. Another possibility is that the treatment caused some peculiar aggregation of otherwise intact virus so that on subsequent density gradient centrifugation a localized overloading of the gradient occurred and the material migrated to an anomalously high density. A third possibility is that the ether-detergent treatment caused a rupture of the viruses to subviral constituents.

The additional experimental evidence appears to eliminate the first two hypotheses and to be in accord with an interpretation requiring rupture of the viruses.

The density gradient behavior of the aqueous extract and ether extract produced from these murine leukemia viruses was similar to that of corresponding extracts prepared by ether-detergent treatment of influenza virus and NDV (*9*). The rupture of various myxoviruses on exposure to ether has previously been demonstrated by electron microscopy (*5*), immunological methods (*22*), and chemical analyses (*23*). The equilibrium density position of the materials in the aqueous extracts of these leukemia viruses was typically that of nucleoprotein material and quite different from that expected of lipid-rich, membrane-enclosed virions. Furthermore, the electron microscopy studies revealed the absence of any intact, membrane-enclosed virions in such preparations and showed, instead, new particulate structures. The gradient purified materials obtained from the aqueous extract failed to react in the micro Ouchterlony immunodiffusion test with immune sera prepared against intact virus and were thus free of detectable exterior viral antigen, whereas both the untreated virus and the ether extract did react.

The aqueous extracts obtained with the Rauscher and Moloney viruses contained structures that, although lacking in viral membrane, still showed a considerable degree of structural complexity and organization: Possibly, these structures may be essentially equivalent to the nucleoids observed as more intensely staining inner components in preparations of the intact virions. Whatever their precise nature, clearly the ultimate constituent units of the Rauscher and Moloney murine leukemia viruses are not attained by Tween-ether or sodium deoxycholate treatment of the virions. Other procedures aimed at sequential unfolding of the structures are now being explored.

SUMMARY

The rupture of the Rauscher and Moloney murine leukemia viruses to subviral constituents on treatment with ether, together with one of the detergents Tween 80 or sodium deoxycholate, is described. Centrifugation of the intact Rauscher virus on sucrose or potassium citrate density gradients yielded a single 1.16 density band containing the viral infectivity. Band isolates from sucrose gradients gave specific precipitation when reacted with antisera prepared against the Rauscher virus in a micro Ouchterlony immunodiffusion experiment and specifically fixed complement in the presence of sera obtained either from rats carrying a Rauscher virus-induced tumor or from BALB/c mice immunized with

formalin-treated Rauscher virus. Density gradient examination of the products obtained on treatment of the Rauscher virus with Tween-ether or sodium deoxycholate-ether showed the absence of normal density virus and the generation of both very low density and higher density (1.24–1.27) components. Electron microscopic examination of the new high density constituents revealed structures devoid of viral membrane, tentatively identified as viral nucleoids.

REFERENCES

(*1*) Old, L. V., and Boyse, E. A.: Immunology of experimental tumors. Ann Rev Med 15: 167–186, 1964.

(*2*) Bryan, W. R., Moloney, J. B., O'Connor, T. E., and Fink, M. A.: Viral etiology of leukemia. Ann Intern Med 62: 376–398, 1962.

(*3*) O'Connor, T. E.: Murine leukemia viruses: A review. Presented at workshop on prospects for control of viral-induced tumors by immunological methods and chemotherapy held by the subcommittee on Carcinogenesis and Prevention of the National Cancer Advisory Council 6/18/65. To be published in the workshop volume, University of Utah Press.

(*4*) Schafer, W.: Structure of some animal viruses and significance of their components. Bact Rev 27: 1–17, 1963.

(*5*) Horne, R. W., and Waterson, A. P.: A helical structure in mumps, Newcastle disease and Sendai viruses. J Molec Biol 2: 75–77, 1960.

(*6*) Hoyle, L., Horne, R. W., and Waterson, A. P.: The structure and composition of myxoviruses. II. Components released from the influenza virus particle by ether. Virology 13: 448–459, 1961.

(*7*) Eckert, E. A., Rott, R., and Schafer, W.: Studies on BA1 strain A (avian myeloblastosis) virus. I. Production and examination of potent virus-specific complement-fixing antisera. Virology 24: 426–433, 1964.

(*8*) ———: Studies on the BA1 strain A (avian myeloblastosis) virus. II. Some properties of viral split products. Virology 24: 434–440, 1964.

(*9*) O'Connor, T. E., de-Thé, G., Rauscher, F. J., and Fink, M.: Murine leukemia viruses and myxoviruses: Comparison of rupture of these viruses with ether and detergents. A preliminary report. Presented at workshop on prospects for control of viral-induced tumors by immunological methods and chemotherapy held by the subcommittee on Carcinogenesis and Prevention of the National Cancer Advisory Council 6/18/65. To be published in workshop volume, University of Utah Press.

(*10*) de-Thé, G., and O'Connor, T. E.: Structure of a murine leukemia virus after disruption with Tween-ether. Virology 28: 713–728, 1966.

(*11*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*12*) Dalton, A. J., and Moloney, J. B.: Recovery of virus from the blood of rats with induced leukemia. *In* Symposium: The Interpretation of Ultrastructure (Harris, R. J. C., ed.). New York, Academic Press Inc., 1962, pp 385–392.

(*13*) O'Connor, T. E., Rauscher, F. J., and Zeigel, R. F.: Density gradient centrifugation of a murine leukemia virus. Science 144: 1144–1147, 1964.

(*14*) Sabatini, D. C., Bensch, K., and Barrnett, P. J.: Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol 17: 19–58, 1963.

(*15*) Mollenhauer, H. H.: Plastic embedding mixtures for use in electron microscopy. Stain Techn 39: 111–114, 1964.

(*16*) Watson, M. L.: Staining of tissue sections for electron microscopy with heavy metals. J Biophys Biochem Cytol 4: 475–478, 1958.

(*17*) Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212, 1963.

(*18*) Wasserman, E., and Levine, L. J.: Quantitative microcomplement fixation and its use in the study of antigenic structure by specific antigen-antibody inhibition. J Immun 87: 290–295, 1961.

(*19*) Gerber, P., and Birch, S. M.: Antigenic analysis of virulent and attenuated type I polioviruses by quantitative complement fixation. J Immun 95: 70–74, 1965.

(*20*) Osler, A. G., Strauss, J. H., and Mayer, M. M.: Diagnostic complement fixation. I.A. method. Amer. J Syph 36: 140–153, 1952.

(*21*) Fink, M. A., and Rauscher, F. J.: Immune reactions to a murine leukemia virus. I. Induction of immunity to infection with virus in the natural host. J Nat Cancer Inst 32: 1075–1082, 1964.

(*22*) Lief, F. S., and Henle, W.: Studies on the soluble antigen of influenza virus I. The release of S antigen from elementary bodies by treatment with ether. Virology 2: 753–771, 1956.

(*23*) Frisch-Niggemeyek, W., and Hoyl, L.: The nucleic acid and carbohydrate content of influenza virus A and of various fractions produced by ether disintegration. J Hyg (Camb) 54: 201–212, 1956.

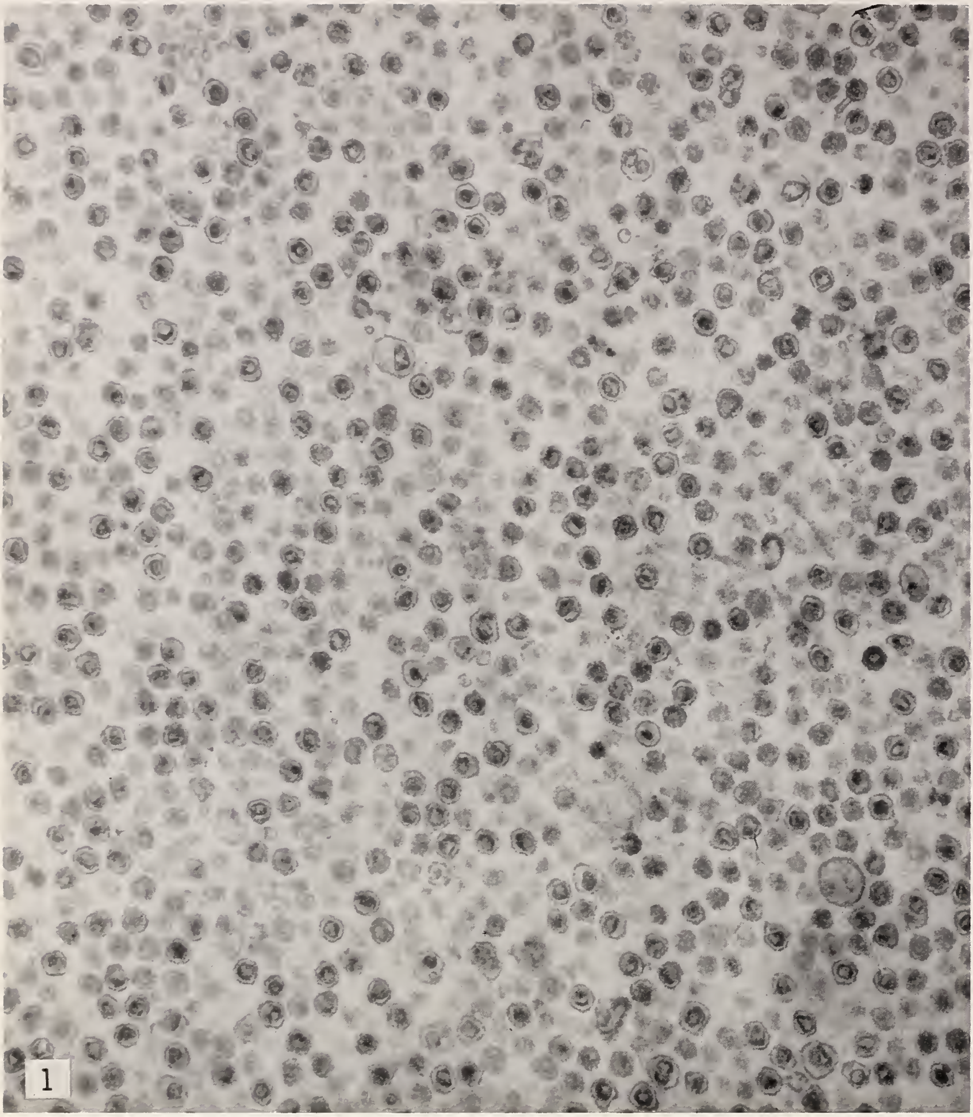
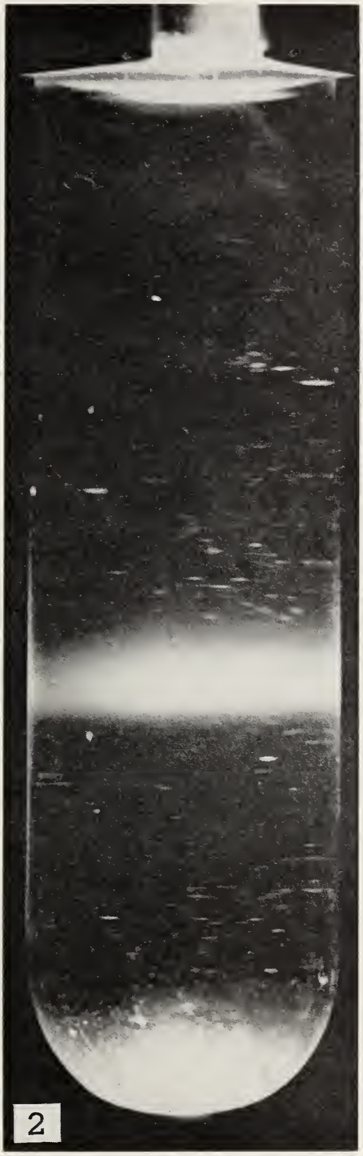


FIGURE 1.—Thin section of a Rauscher virus pellet. × 32,000



2

FIGURE 2.—Density gradient centrifugation of Rauscher virus on sucrose gradient. Virus overlaid on preformed gradient and centrifuged 4 hours on SW39L rotor at 35,000 rpm.

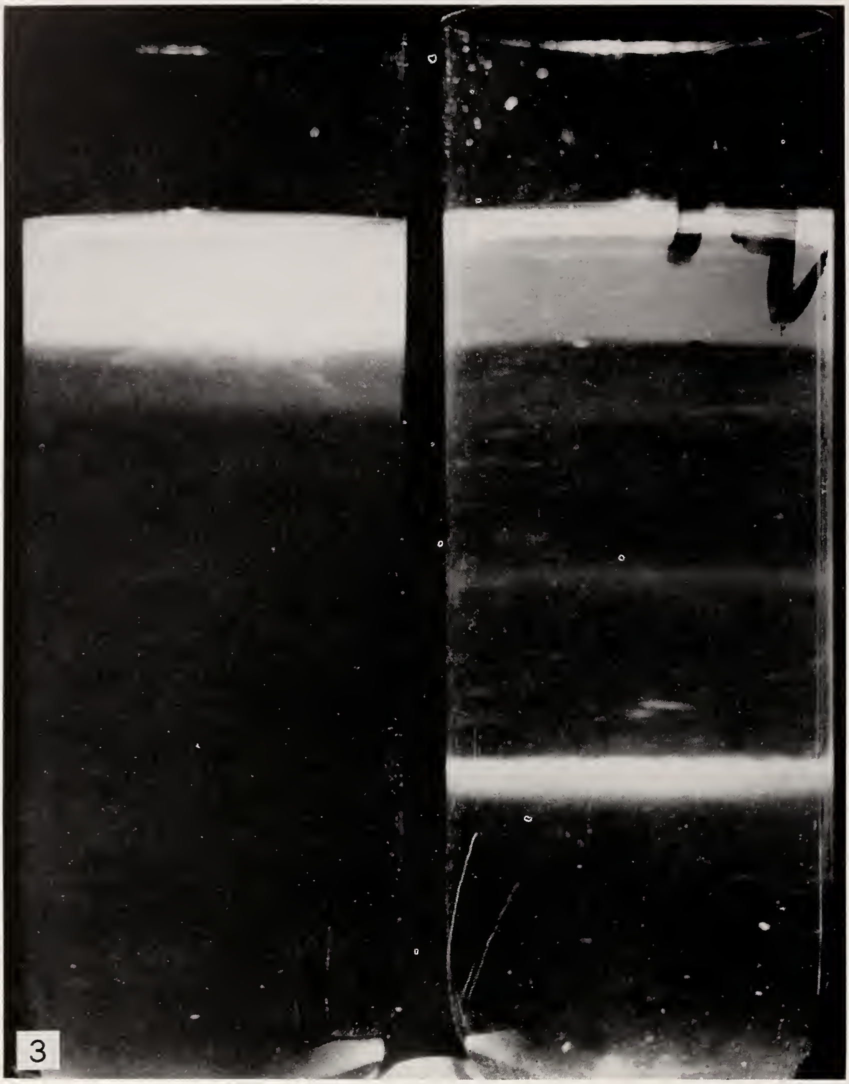

3

Figure 3.—Density gradient centrifugation of Tween 80-ether-treated Rauscher virus products on potassium citrate gradients. *Left tube:* "ether extract"; *right tube:* "aqueous extract." Materials overlaid on identical preformed gradients and simultaneously centrifuged for 3 hours on SW25-1 rotor at 22,500 rpm.

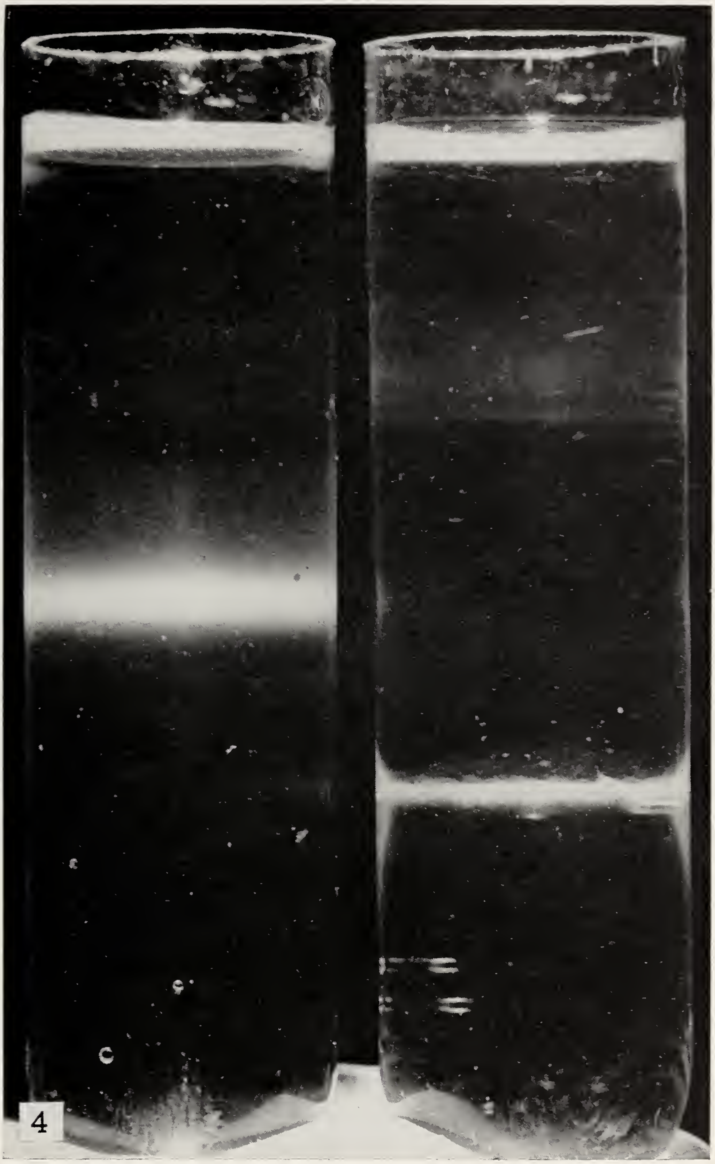


FIGURE 4.—Density gradient centrifugation of untreated Rauscher virus (*left tube*) and of "aqueous extract" (*right tube*) after sodium deoxycholate-ether treatment of the virus. Materials were simultaneously centrifuged for 2 hours on identical potassium citrate gradients on SW39L rotor at 35,000 rpm.

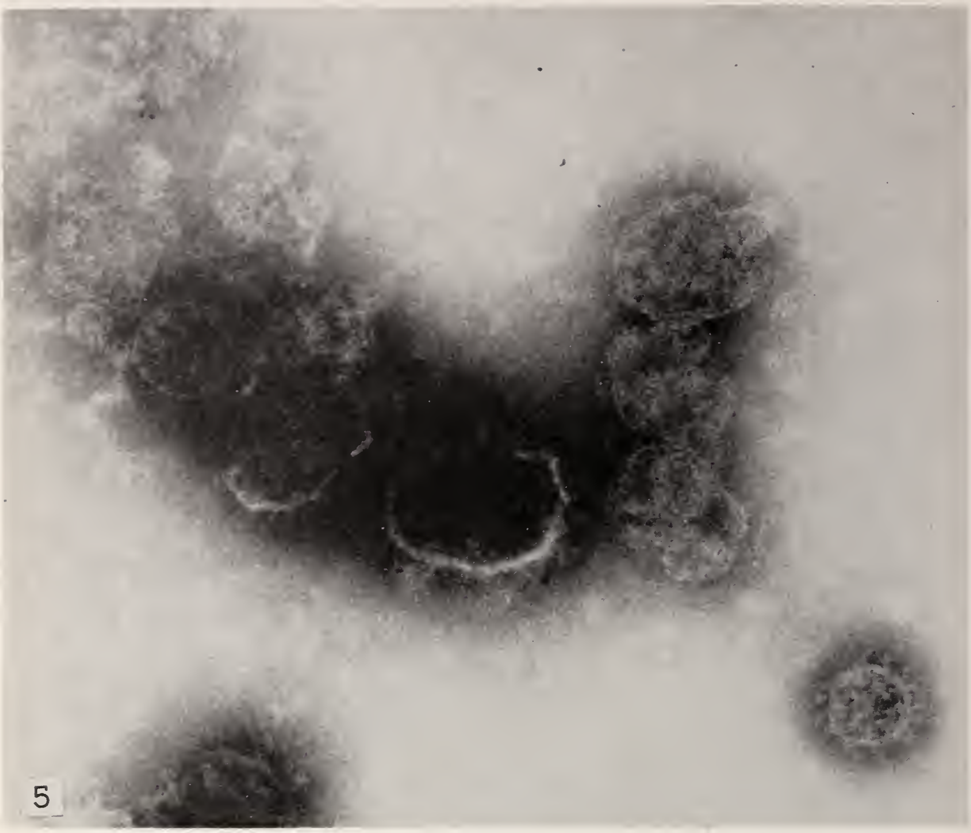


FIGURE 5.—Negative stained preparation of resuspended pellet (P2) from the "aqueous extract" of Tween-ether-treated Rauscher virus. Dense spherical bodies, 700 to 750 A in diameter are evident. × 215,000

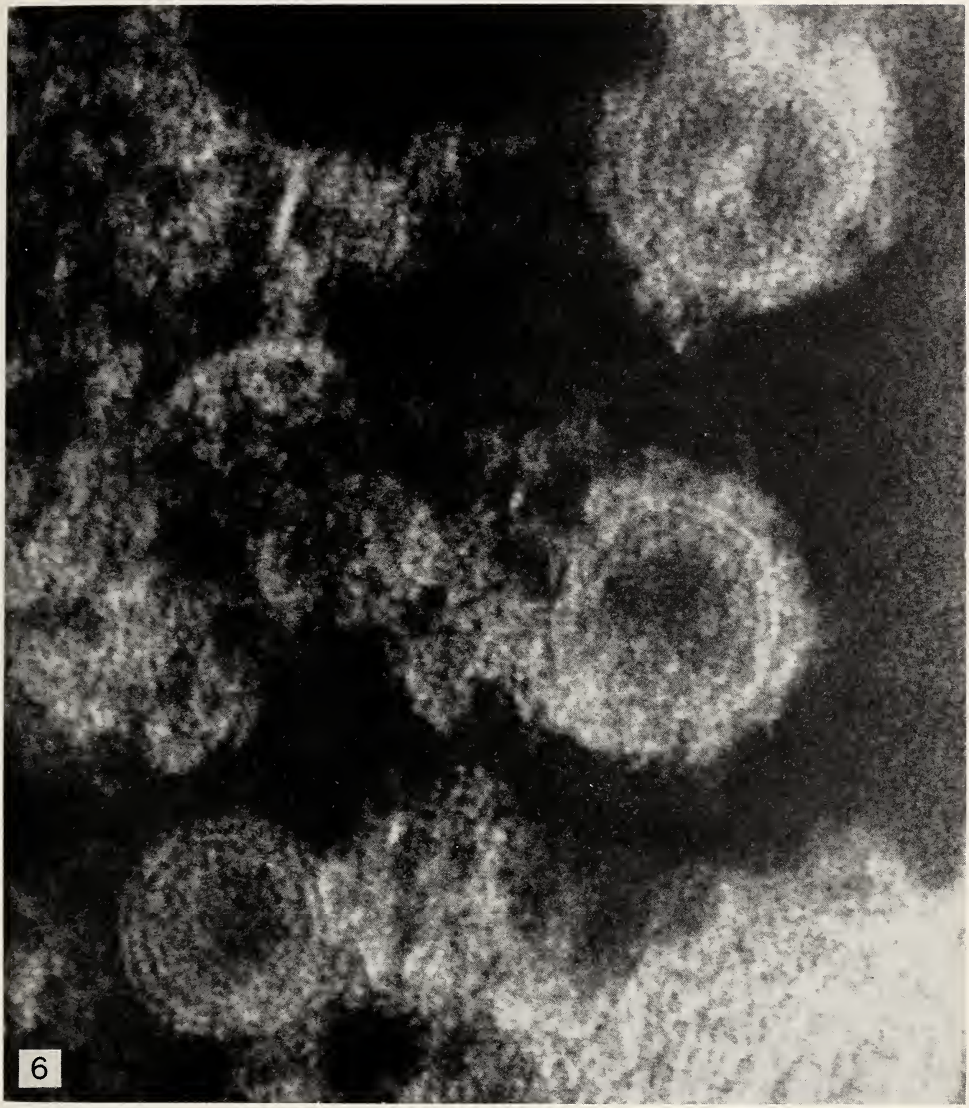


FIGURE 6.—Same preparation as figure 5, where ringlike profiles are evident. The rings are 75 A thick, have two apparent walls 25 to 30 A thick and a hollow 15 to 25 A core and are 750 to 850 A in over-all diameter. × 450,000

Studies on Leukemia in Mice Associated With a Transplantable Methylcholanthrene-Induced Hepatoma [1, 2]

L. Dmochowski, T. Rogers, A. G. Liebelt, R. A. Liebelt, and J. A. Sykes, *Department of Virology, The University of Texas M. D. Anderson Hospital and Tumor Institute, and Departments of Microbiology and Anatomy, Baylor University College of Medicine, Texas Medical Center, Houston, Texas*

TUMORIGENESIS in the mouse is known to be dependent on multiple factors: genetic, hormonal, and viral. The relationship of a primary or transplanted tumor to the subsequent development of other types of neoplasms must also be considered. Recently we reported (*1*) that leukemia developed in mice in which transplants from a methylcholanthrene-induced hepatoma either regressed or were surgically removed. Similarly, leukemia was found by Handler (*2*) and by Sproul (*3*) in mice bearing transplanted pituitary tumors. Transplantable tumors have been found to be the source of several leukemogenic viruses [reviewed by Dmochowski (*4*)]. The etiology and nature of this relationship are being studied in the light of the occasional reports in the literature of leukemia and cancer in the same patient.

MATERIALS AND METHODS

The strain CBA mice used in these studies were from the inbred line maintained in the Department of Anatomy, Baylor University College of Medicine. The original hepatoma arose in a female CBA mouse that had been fed 9 doses of 1.0 mg of 3-methylcholanthrene in olive oil. This hepatoma formed the basis for the present series of experiments

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported in part by Public Health Service research grants CA–04140–06 and CA–07914–01 from the National Cancer Institute, and by grant E–94–E from the American Cancer Society Inc.

during subsequent transplant generations. The hepatoma was transferred to 4- to 9-day-old CBA mice from the same colony by subcutaneous inoculation of tumor cell mince in normal saline. Of the 7 mice used for the first transplant generation, 3 developed continuously growing subcutaneous hepatomas. In the remaining 4 mice, hepatomas grew progressively for 2 to 3 months, reaching an approximate diameter of 1 cm, after which the tumors regressed. Within 198 to 253 days after transplantation these mice had hepatosplenomegaly. The leukocyte count varied between 60,000 and 120,000 per mm^3, and peripheral blood smears were suggestive of a monocytic leukemia (fig. 1). Examination of the original hepatoma (fig. 2) revealed no foci of leukemic cell infiltration.

In the second transplant generation the hepatoma grew in 3 of 16 mice, none of which showed any signs of hepatosplenomegaly or leukemia. They were killed at 311 days. The remaining 13 mice showed only temporary growth of the hepatoma transplants and all developed hepatosplenomegaly and a blood picture of monocytic leukemia between 171 and 311 days. In the third transplant generation the hepatoma grew well in all the inoculated mice and none developed leukemia. In the fourth transplant generation the hepatomas in 4 mice were removed surgically 123 days after the transplant; all 4 developed hepatosplenomegaly and leukemia 137 days later. The remaining tumor-bearing hosts died free from leukemia at 240 days. In the fifth transplant generation, the tumor grew in all inoculated mice and none had leukemia at death 230 days after the transplant. The incidence of spontaneous reticular neoplasms in more than 500 control CBA mice over the past 5 years has been less than 3%.

In an attempt to study this unusual relationship, between a transplanted hepatoma and the occurrence of hepatosplenomegaly associated with a blood picture indicative of monocytic leukemia, hepatomas in subsequent transplant generations were removed surgically and the mice observed for development of the characteristic hepatosplenomegaly and associated blood picture.

Tumor transplants were made from hepatomas removed surgically from these CBA mice by finely mincing the tumor and suspending the mince in normal saline for subcutaneous inoculation into 4- to 9-day-old CBA mice.

Cell-free preparations of transplanted hepatomas or leukemic spleens were made by first finely mincing the tissues and then grinding the tissue mince in a chilled mortar with sterile sand. The ratio of suspending fluid to weight of tissue was 20:1. Hanks' balanced salt solution (BSS) was used as diluent. The homogenate was clarified by centrifugation at $1000 \times g$ for 30 minutes at 4° C and the supernatant fluid further clarified by centrifugation at $7000 \times g$ for 15 minutes at 4° C. This cell-free material was used for intraperitoneal inoculation of 4- to 9-day-old CBA mice. They were weaned at 3 weeks of age and observed for 18 months, at which time they were killed and autopsied.

Tissue cultures of the surgically removed transplanted hepatomas and of spleens from leukemic mice were maintained in T-30 culture flasks. The minced tissues were treated with 0.25% trypsin in Hanks' BSS and cultured in a modified complete Eagle's medium containing 20% calf serum with 0.5 neomycin and 0.125 g streptomycin per liter of medium. Cultures were incubated at 38° C and were subcultured when cell growth and acidity of the medium indicated. At regular intervals cells were also cultured in Sykes-Moore culture chambers (*5*) for phase-contrast microscopic examination and photography.

Tissue culture preparations used for inoculation of CBA mice were prepared in the following manner. The cells were sonic vibrated for 1 minute with a Branson Sonifier at a setting of 2.8 amps. The mixture of cell debris and tissue culture medium was clarified in a similar manner to that used for the tissue homogenates and the cell-free material inoculated in the same way into 4- to 9-day-old CBA mice. These mice were also observed for 18 months.

Tissues and tissue culture cells were prepared for electron microscopy by fixation in phosphate-buffered osmium tetroxide, according to the method of Millonig (*6*), at 4° C for 18 hours. After thorough washing of the fixed material with phosphate buffer, the tissue was dehydrated by use of a graded series of ethanol concentrations, then embedded in prepolymerized butyl methacrylate, and finally polymerized in a vacuum oven at 56° C. Sections for electron microscopy were cut at a thickness of 400 A with a Servall automatic ultramicrotome. Sections were collected on collodion-coated 200-mesh stainless steel screens, stained for 2 hours with uranyl acetate, and then counterstained for 10 minutes with lead tartrate. A thin coating of carbon was evaporated over the sections before examination in the electron microscope. They were examined either in an RCA EMU 3-E or an EMU 3-G electron microscope at a magnification varying from 7,000 to 20,000 times.

RESULTS

Cell-free homogenates of hepatomas, leukemic spleens, or tissue culture cells were used for animal inoculation studies. More than 300 CBA mice were inoculated (11 experiments) with extracts of hepatomas or of leukemic spleens. During the 18 months of observation no tumors were noted. There was no evidence of disease at autopsy. Tissue culture preparations derived from hepatomas and from leukemic spleens were used in a series of 14 experiments involving more than 350 CBA mice. Mice were observed for 18 months before autopsy following etherization. During this period no evidence of tumors or leukemia was seen and at autopsy the mice appeared healthy.

Tissue culture studies included growth of cells derived from transplanted hepatomas removed surgically or obtained at autopsy, and from leukemic spleens and livers of mice showing hepatosplenomegaly associated with these hepatomas. A total of 25 hepatoma cell cultures were made and maintained for periods varying from 4 to 8 weeks, with a total number of passages varying from 1 to 7. Cells were characteristically epithelioid (fig. 3) and when examined by phase contrast microscopy showed no evidence of cytopathic changes or of cytoplasmic inclusions.

In 11 experiments, cell cultures were prepared from spleens of mice showing hepatosplenomegaly associated with removal of or regression of the transplanted hepatoma. Figure 4 shows the histologic pattern seen in sections of the liver from such mice. The pattern of leukemic cell infiltration differs from that seen with some of the murine leukemia viruses, such as Gross, Moloney, Friend, and Rauscher. The cell cultures of leukemic spleens were maintained for a maximum of 5 passages over a period of 6 weeks. The average time in culture was 4 weeks. Study of cells of these cultures by phase contrast microscopy revealed no characteristic changes; cytopathic changes were not observed nor was there any evidence of cytoplasmic or other inclusions. The growth pattern of these cells was indistinguishable from that exhibited by the cells derived from the transplanted hepatomas (fig. 3).

Examination of tissue culture preparations derived from transplanted hepatomas or leukemic spleens, stained with an acridine (Coriphosphin O) and examined in the fluorescence microscope, revealed no unusual concentrations of RNA or DNA within the cells.

Electron microscope studies of the hepatomas removed surgically or at autopsy were carried out on different transplant generations of the original tumor. Examination of ultrathin sections taken from tumors in early transplant generations revealed the occasional budding of virus particles from plasma membranes (fig. 5). Rarely, small groups of mature virus particles resembling those associated with murine leukemias were observed. In later transplant generations of the hepatoma, virus particles were more frequent and were present in larger aggregates. In some instances there were virus particles within membranous structures in the cytoplasm, and the number of extracellular virus particles had increased (fig. 6).

Cells derived from different transplant generations of the hepatoma were grown in tissue culture and examined in the electron microscope after a varying number of passages *in vitro*. A striking feature of the hepatoma cells in tissue culture was that after only a short time *in vitro* (4 days) mature virus particles could be seen lying along the plasma membranes and between cells (fig. 7). Examination of cells from later tissue culture passages revealed an apparent increase in the number of mature virus particles with passage. Tissue culture cells derived from later transplants of the hepatoma had more virus particles than tissue cultures derived from early transplants of the hepatoma in a similar number of tissue culture passages (fig. 8).

As has been mentioned, when the hepatoma transplants were removed surgically or regressed spontaneously after reaching about 1 cm in diameter, marked hepatosplenomegaly developed within 200 days after the transplant had been made. In electron micrographs of different transplant generations of the leukemic spleens, virus particles similar to those seen in the virus-induced murine leukemias were present. Virus particles could be seen budding from plasma membranes and into cytoplasmic vacuoles (fig. 9) and were frequently observed in the intercellular spaces (fig. 10). Granular osmiophilic inclusions limited by a double membrane and an increased number of virus particles within cytoplasmic vacuoles were seen in later transplants (fig. 11).

Cells derived from different transplant generations of leukemic spleens were grown in tissue culture and examined in the electron microscope. Only small differences in the number of virus particles were seen between cells derived from early and late transplant generations. Virus particles were observed and their numbers appeared to increase in the later transplants. Cells from early tissue culture passages seemed to have fewer virus particles than later passages, but an accurate evaluation would be difficult owing to the high sampling error associated with electron microscope studies of thin sections. Budding of virus particles from plasma membrane was frequently observed (fig. 12), and virus particles were also seen within cytoplasmic vacuoles and lying along plasma membranes (fig. 13).

DISCUSSION

The association of a transplantable methylcholanthrene-induced hepatoma with hepatosplenomegaly and monocytic leukemia was originally reported by Leibelt *et al.* (*1*). A preliminary electron microscope study of the induced and transplanted hepatoma and of the associated leukemia was reported by Dmochowski *et al.* (*7*).

Histologically, the hepatomas are well-differentiated tumors resembling normal liver. Electron microscope studies of spontaneous, induced, and transplanted hepatomas in mice of different strains have recently been reported by Dmochowski *et al.* (*8*). Only quantitative differences were observed between nuclear and cytoplasmic constituents of hepatoma and normal liver cells. Cytoplasmic inclusions, varying in size up to 0.4 μ surrounded by one or two membranes, uniformly electron-dense, have been observed in methylocholanthrene-induced hepatomas in CBA mice. In addition, occasional immature murine leukemia virus particles and their budding from plasma membranes have been observed in the original methylcholanthrene-induced hepatomas.

In the present study, both the induced hepatomas following transplantation and transplanted leukemic spleens from mice with leukemia associated with the hepatomas were examined in the electron microscope before and after passage in tissue culture. Only few immature virus particles were found in the early transplant generations of the induced hepatoma.

Mature virus particles have been found in cells of these hepatomas after tissue culture passage, increasing in number in later tissue culture passages. There was an increase in immature and mature leukemia-type virus particles in later transplant generations of the hepatoma. Again growth in tissue culture of cells from these hepatomas increased even more the number of virus particles. Similar findings have been obtained in the electron microscope studies of leukemic spleens on transplantation and after tissue culture. These findings suggest that tissue culture passage may remove a hypothetical inhibitor of virus multiplication.

Despite the morphological observations, no biological information about the nature of the leukemia virus-like particles has so far been obtained. This may be due entirely to insufficient titer of the virus particles present in the different cell-free preparations examined. Additional bioassays are in progress with more concentrated material and in mice of other strains known to be susceptible to virus-induced leukemia.

The occurrence of a monocytic leukemia blood picture with hepatosplenomegaly has not previously been reported associated with hepatomas. It is not known at present what may be the parts played by methylcholanthrene and by the induced hepatomas in the origin of this type of leukemia.

SUMMARY

Transplantation of a hepatoma induced in a strain CBA mouse by feeding methylcholanthrene was found to be associated with hepatosplenomegaly and monocytic leukemia when transplants of the hepatoma in CBA mice regressed or were removed surgically. Transplantation of cells from the enlarged (leukemic) spleens resulted in the induction of monocytic leukemia in the host CBA mice. Hepatomas and leukemic spleens from different transplant generations in CBA mice were examined in the electron microscope. They were also grown in tissue culture. Cells from different tissue culture passages derived from the transplanted hepatomas and from the transplanted leukemic spleens were also examined in the electron microscope. Electron microscopy of the transplanted hepatomas demonstrated the presence of virus particles similar to murine leukemia virus particles and an increase in the numbers of these particles after repeated transplants of the hepatomas. Electron microscopy of leukemic spleens from different transplant generations of leukemia in CBA mice showed the presence of characteristic virus particles within cells and budding from the plasma membranes; particles increased with successive transplants. Examination of cells derived from tissue cultures of hepatomas and of spleens showed increase in the number of mature virus particles following tissue culture compared with the number in the original tissue. The number of virus particles increased after successive tissue culture passages. The relationship of the observed virus particles to the origin of the monocytic leukemia is being studied.

REFERENCES

(*1*) LIEBELT, R. A., LIEBELT, A. G., DMOCHOWSKI, L., and SYKES, J. A.: Leukemia in CBA mice after transplantation of a methylcholanthrene (MC)-induced hepatoma. Proc Amer Assoc Cancer Res 4: 38, 1963.

(*2*) HANDLER, A. H., and WILLS, C.: Mouse leukemia resulting from the implantation of an ACTH-secreting pituitary tumor. Fed Proc 22: 605, 1963.

(*3*) SPROUL, E. E., GRINBERG, R., and WERNER, S. C.: Lymphoid hyperplasia and neoplasia associated with a mouse pituitary thyrotropic tumor. Cancer Res 23: 1090–1096, 1963.

(*4*) DMOCHOWSKI, L.: The viral etiology of leukemia. Progr Med Virol 3: 363–494. 1960.

(*5*) SYKES, J. A., and MOORE, E. B.: A simple tissue culture chamber. Texas Rep Biol Med 18: 288–297, 1960.

(*6*) MILLONIG, G.: Advantages of a phosphate buffer for OsO_4 solution in fixation. Proc 19th Ann Meeting Electron Microscopy Soc America, 1961, pp 15–16.

(*7*) DMOCHOWSKI, L., PADGETT, F., SYKES, J. A., LIEBELT, R. A., and LIEBELT, A. G.: Electron microscopy of a methylcholanthrene-induced (MC) hepatoma and an associated leukemia. Proc Amer Assoc Cancer Res 4: 16, 1963.

(*8*) DMOCHOWSKI, L., ROGERS, T., SYKES, J. A., LIEBELT, R. A., and LIEBELT, A. G.: Studies on spontaneous, induced, and transplanted hepatomas in mice of different inbred strains and their hybrids. Proc 23d Ann Meeting Electron Microscopy Soc America, 1965, p 6.

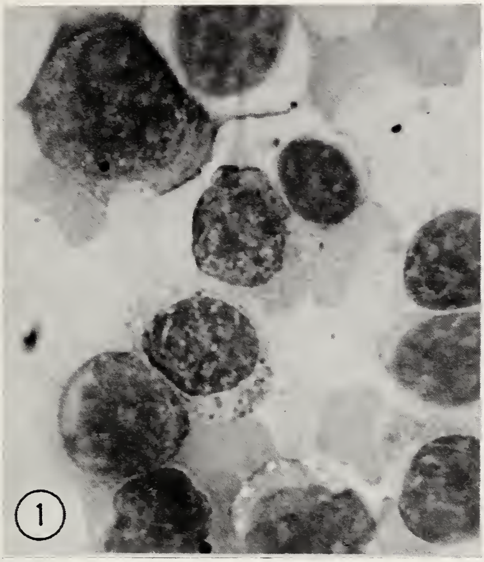
Plate 60

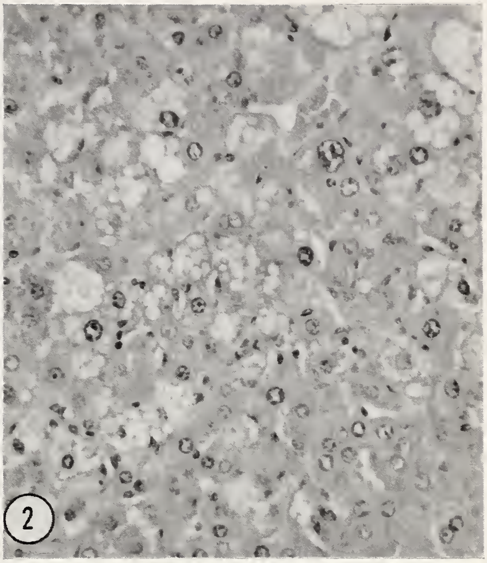
Figure 1.—Peripheral blood smear stained with May-Grünwald-Giemsa from a strain CBA mouse carrying the first transplant generation of the methylcholanthrene-induced hepatoma after regression of the transplant. *Note* characteristic primitive monocytes associated with the leukemia. × 1,200

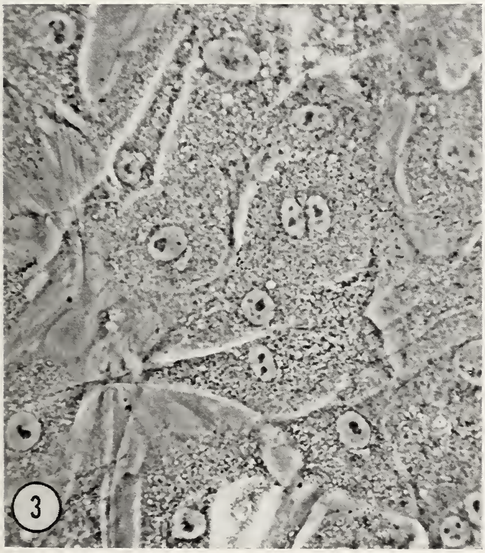
Figure 2.—Portion of methylcholanthrene-induced hepatoma from a CBA mouse. Hematoxylin and eosin. × 210

Figure 3.—Phase contrast photomicrograph of cells growing in tissue culture derived from the seventh transplant generation of the original methylcholanthrene-induced hepatoma. × 450

Figure 4.—Section of liver from a CBA mouse carrying the 24th transplant generation of the monocytic leukemia associated with the methylcholanthrene-induced transplantable hepatoma. Hematoxylin and eosin. × 210


1


2


3

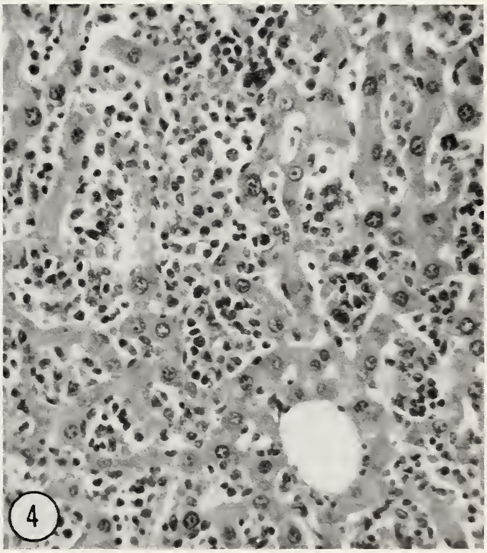

4

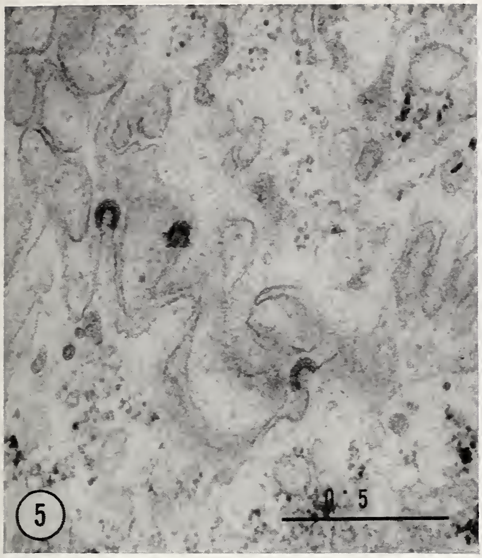
Plate 61

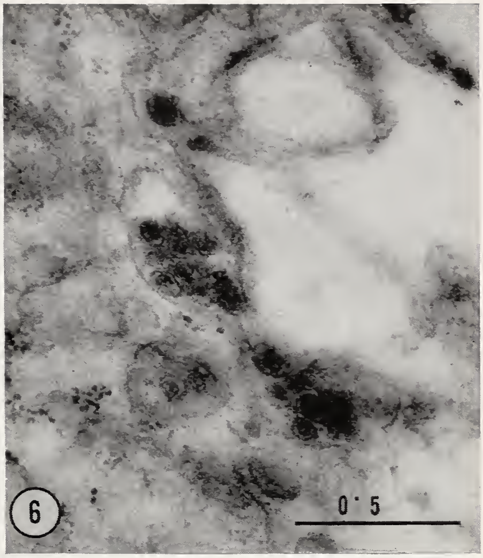
Figure 5.—Electron micrograph of a portion of a hepatoma from the sixth transplant generation showing budding of virus particles from plasma membranes. × 46,500

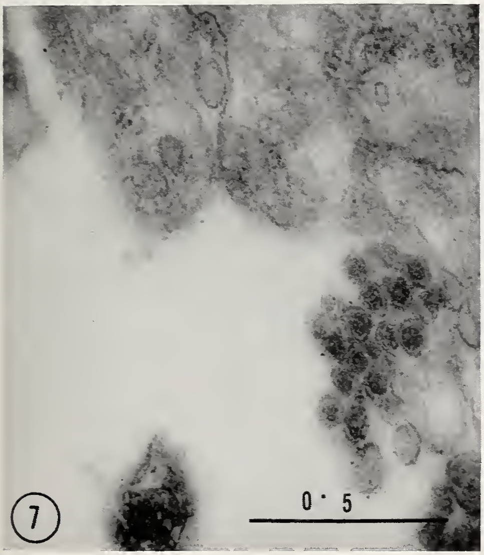
Figure 6.—Electron micrograph of a portion of a hepatoma from the sixth transplant generation showing virus particles within a membrane-bounded vesicular structure of the cytoplasm. × 46,500

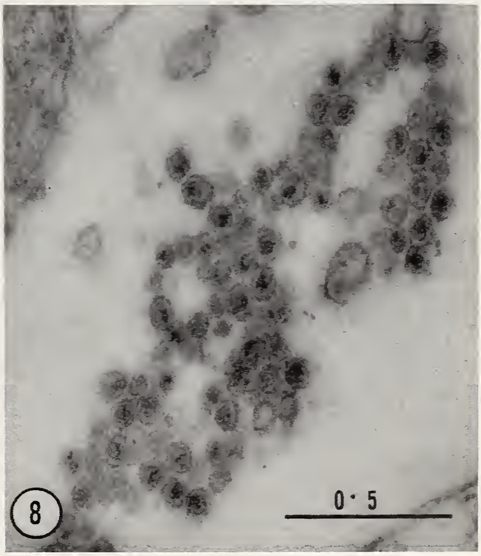
Figure 7.—Electron micrograph of tissue culture cells derived from the sixth transplant generation of the hepatoma during the first passage *in vitro*. *Note* mature virus particles lying along the plasma membrane. × 45,000

Figure 8.—Electron micrograph of tissue culture cells in passage 4 from the sixth transplant generation of the hepatoma. A large group of mature virus particles lying outside the cells may be seen. × 54,100

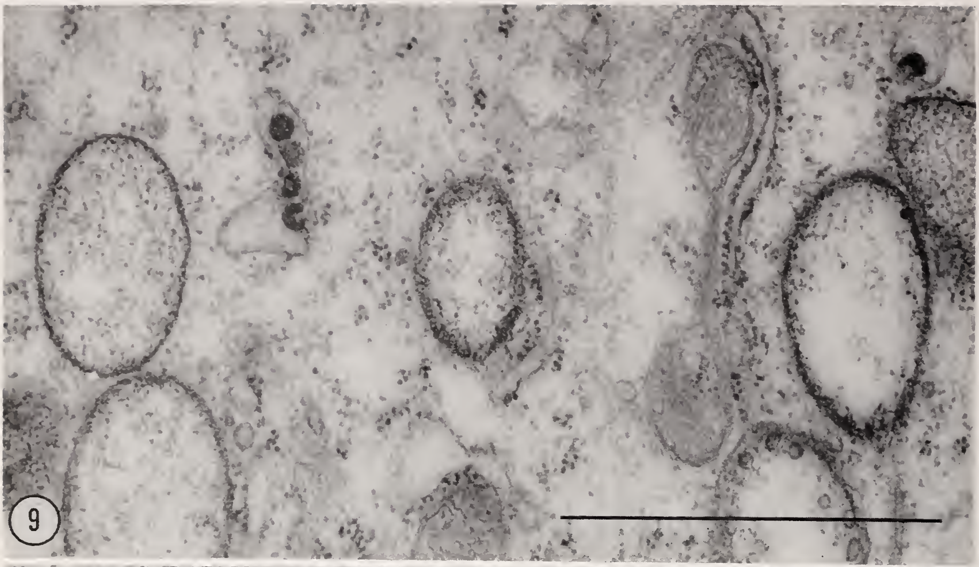

⑤


⑥


⑦


⑧

9



10

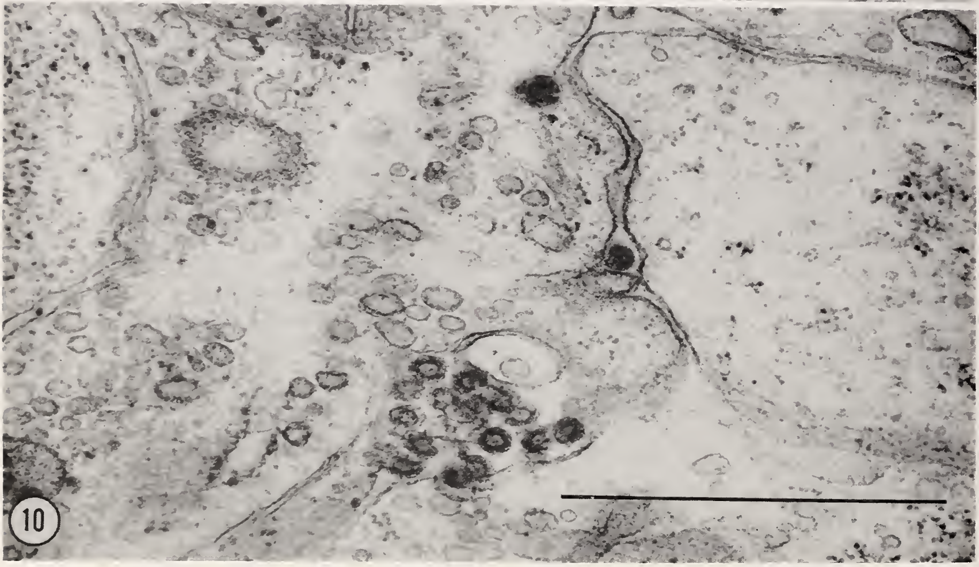
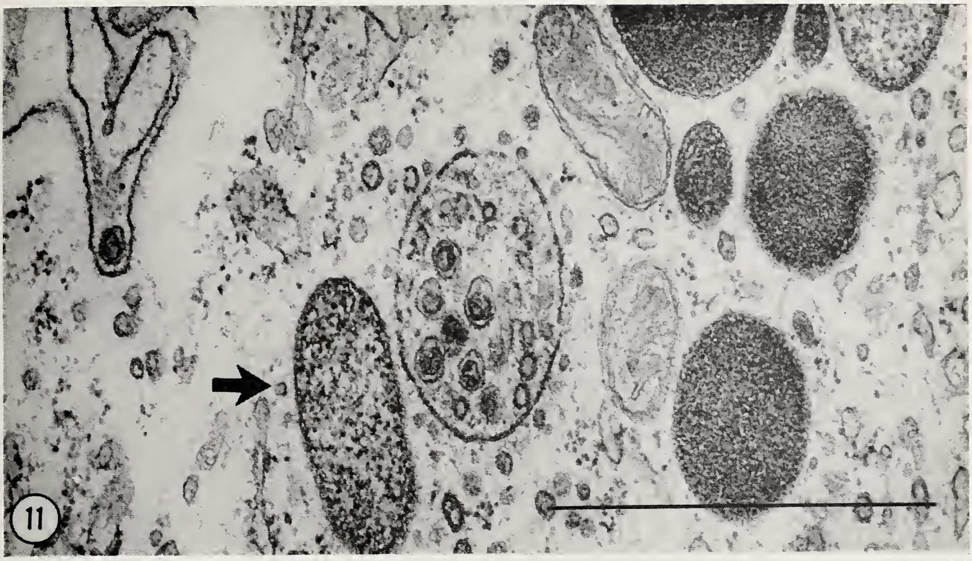


FIGURE 9.—Electron micrograph of a portion of a leukemic spleen from the third transplant generation showing budding of virus particles into an intracytoplasmic vacuole. × 52,500

FIGURE 10.—Electron micrograph of a portion of a leukemic spleen from the third transplant generation showing budding and a group of immature virus particles lying between the plasma membranes of two adjacent cells. × 52,500



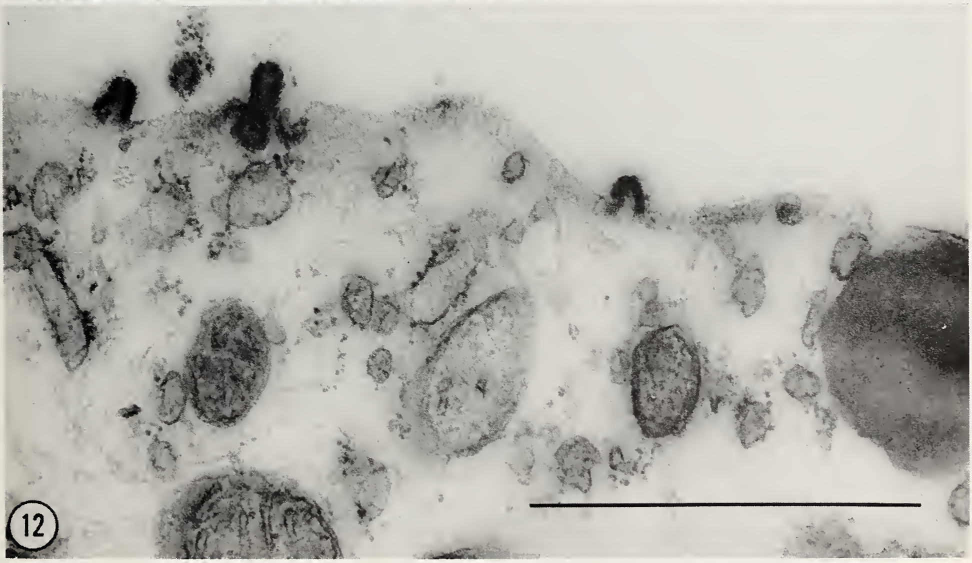


FIGURE 11.—Electron micrograph of a portion of a leukemic spleen from the sixteenth transplant generation showing a cytoplasmic inclusion containing a number of mature virus particles. *Note* granular osmiophilic inclusions surrounded by double membranes and also a structure resembling mycoplasma (*arrow*). × 60,000

FIGURE 12.—Electron micrograph of a cell from the third tissue culture passage of a leukemic spleen of the fourth transplant generation. Different stages of virus particles budding from the plasma membrane are visible. × 60,000

13

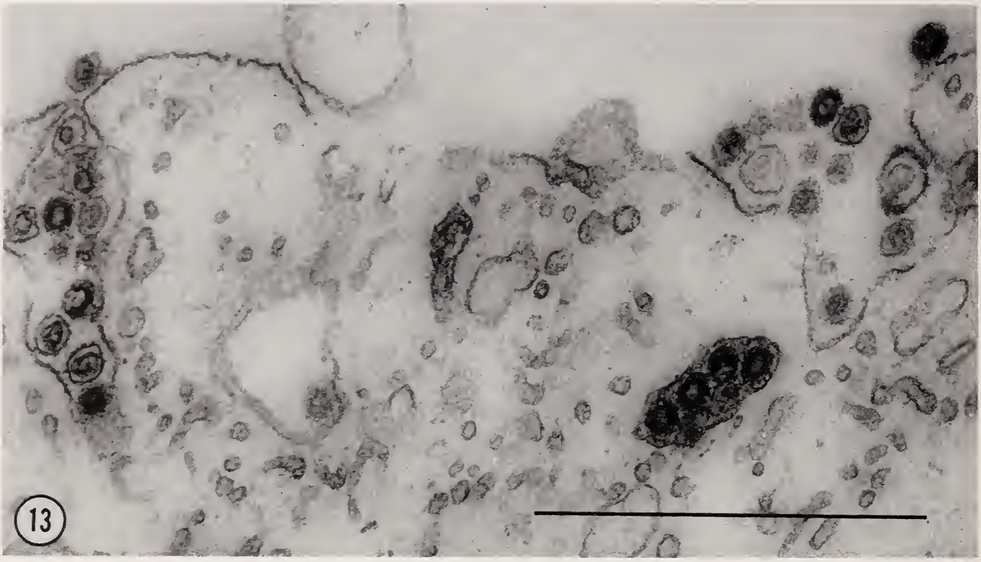


FIGURE 13.—Electron micrograph of a portion of a cell from the third tissue culture passage of a leukemic spleen from the fourth transplant generation. Mature virus particles are adjacent to the plasma membrane and some are apparently being extruded from the cell through a cytoplasmic vacuole. A small cytoplasmic inclusion containing 4 immature virus particles is also visible. × 60,000

Observations on the Morphology of a Murine Leukemia Virus (Rauscher) Propagated in Tissue Culture[1]

Robert F. Zeigel, Richard L. Tyndall,[2] Timothy E. O'Connor, Ernestine Teeter,[2] *and* Barbara V. Allen,[3] *Laboratory of Viral Oncology, National Cancer Institute,*[4] *Bethesda, Maryland, and Biology Division,*[5] *Oak Ridge National Laboratory, Oak Ridge, Tennessee*

Electron microscopic observations on the *in vivo* proliferation of murine leukemia viruses have been reported (*1–8*). Several different cell types are capable of supporting the reproduction of the virus particles, among which are malignant lymphoblasts, megakaryocytes, eosinophils, neutrophils, plasma cells, erythroblasts, and mammary epithelial cells (*9–11*). These observations led to the development of differential and density gradient centrifugation techniques facilitating concentration of these agents from tissues and particularly from blood plasma (*12*). The murine agents have recently been examined with respect to their sensitivity to treatment with biophysical and biochemical procedures correlated with the concurrent alteration in biological activity (*13*). The establishment of cell lines, capable of supporting the growth of the murine agents *in vitro*, promoted controlled studies relating to growth rate, immunological aspects, and response of the virus to environmental alterations (*14*). Tyndall *et al.* reported specific morphological changes in tissue culture cells infected with the Rauscher agent when subcultured in Eagle's basal medium (*15*). The cells showed evidence of rounding, vacuolation, multiple inclusion bodies, and loss of contact inhibition. These observations prompted an examination of these preparations at the ultrastructural level. The studies reported here had the following purposes:

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

[3] We wish to express our thanks to Mrs. Marian Smith for her very competent assistance during these studies.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[5] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

1) determination of the relationship of these murine leukemia viruses to the cells of their derivation, 2) the mode of viral formation and its association with membranes, 3) the possible inclusion of viral particles in phagosomes, and 4) high resolution analysis of viral internal structure.

MATERIALS AND METHODS

Cell cultures infected with Rauscher virus were propagated in NCTC 109 medium (*15*). These served as controls. For samples for electron microscopy, the cells from several flasks were pooled and packed into a pellet by spinning at 2000 rpm for 15 minutes in a PR–2 (International) centrifuge. These packed pellets were then fixed in Dalton's chrome-osmium for approximately 1½ hours and postfixed in cold, neutral formalin (*16*, *17*). They were then embedded in Epon-Araldite, according to a method modified from Mollenhauer (*18*). Sections were cut with diamond knives on an LKB microtome and double stained with uranyl acetate and lead citrate (*19*). Other preparations of cells subcultured in Eagle's basal medium with 4 times the normal content of amino acids and vitamins (4× BME), which exhibited the specific morphological changes (*15*), were prepared as described. In the hope of showing active phagocytic activity, we infected cell cultures with Rauscher virus growing in 4× BME and inoculated them with unpurified horse-spleen ferritin[6] or with colloidal gold chloride[7] (*20*). The cultures were harvested at several intervals following the addition of these electron-dense markers.

The virus used in the density gradient studies was prepared from the pooled plasmas of inoculated mice and layered on a cesium chloride gradient (*21*). Various sedimentation layers were examined electron microscopically by use of negative staining techniques (*22*, *23*).

OBSERVATIONS

Electron microscopic observations on the Rauscher murine leukemia virus in tissue culture confirmed and extended parallel light microscopic findings (*15*). The infected cells, *in vitro*, resembled most strongly modified fibroblasts and lymphocytes. These cells grown in NCTC 109 medium exhibited a comparatively minimal amount of cytoplasmic vacuolation and relatively little evidence of phagocytosis (figs. 1 and 2). Viral particles were in evidence surrounding the cells and frequently appeared membrane-associated, *i.e.*, in close apposition to the plasma membrane. Immature "C"-type particles, possessing an electron-lucent central region, were occasionally observed. Budding of viral particles was most frequently noted at the periphery of the cells; however, an unusual pattern of viral proliferation was observed (fig. 2). In this instance, many young budding forms are visible originating from the membrane of what is thought to be a developing phagosome. Ribsomal particles were not seen in association with the membrane. A mature phagosome is represented at the left. Mature viral particles are contained within the phagosome. At higher magnification (fig. 3) the virus particles in these cultures were identical in structure to those described in other studies on murine leukemia viruses (*2*, *6–8*). The mature particles were comprised of a peripheral limiting coat, *i.e.*, a unit membrane, derived from the plasma membrane (or similar membrane system in the cell) during the budding process, and a nucleoid region that appears diffuse and granular.

---

[6] Horse-spleen ferritin obtained from Calbiochem, Los Angeles, Calif. (6× crystallized; 0.025–0.05%).

[7] Colloidal gold chloride, obtained from Matheson, Coleman & Bell for Lange's test, East Rutherford, N.J. [The gold was removed by centrifugation and resuspended in solution according to Heine (*20*).]

In thin section, the internal components can best be described as a loosely wound ball of yarn. The immature particles possess a less dense core region, and, in addition, have an intermediate shell somewhat lighter in density than the inner shell. These internal shells "appeared" similar to membrane structures in thin section. There is some evidence, however, from negatively stained preparations that they are not membranes of the usual type, *i.e.*, identical to the plasmalemma. The intermediate shell apparently becomes intimately associated with the material of the inner shell as maturation progresses. The diameter of the nucleoid in mature particles and that of the intermediate shell of the immature particles were essentially the same (about 80 mμ). The diameter of intact mature particles in thin section was approximately 110 mμ. Occasionally, particles with double nucleoids were observed (fig. 4).

The cytological character of cells subcultured in 4× BME was considerably different. Many cells exhibited a highly vacuolated cytoplasm (fig. 5). Large numbers of membrane-associated virus particles were observed. The cytoplasm often contained numerous phagosomes, which enclosed breakdown products of cells and viral particles (fig. 6). Other cells showed evidence of what was assumed to be both viral synthesis (budding) and phagocytosis. From micrographs of these cells grown in 4× BME medium, infected with Rauscher virus, a sequential series of budding forms was constructed (figs. 7 through 14). The continuity of the outer coat with the plasma membrane of the cell was evident. There was little evidence of the presence of spikes or spicules on the exterior of the virus, which corroborated observations on the virus prepared from plasma concentrates in negatively stained preparations (*13*, *23*).

The particles frequently budded from the terminations of cytoplasmic stalks (figs. 8, 9, and 10). The inner components arose as a crescent in thin section and presumably as a cup-shaped structure in three dimensions. The inner and intermediate shells were evident in these immature "C"-type particles. There was an increase in density within the nucleoid region as the particles matured.

Comparison of particles in thin section (figs. 7 through 14) may be made with the structure of the Rauscher virus as revealed by negative staining (fig. 15). Mature and occasionally immature particles were observed. This preparation, from a cesium chloride gradient, permitted the visualization of the internal components against a relatively low-noise background. The coat material was separated from the nucleoid regions in several instances. The nucleoid material appeared as a loosely aggregated meshwork of small (±30 A) subunits arranged in continuous filaments. The periphery of the virus particles was devoid of spikes or knobs. In this connection, it is worthy of note that observations on the Rauscher virus both in thin section and negatively stained preparations have demonstrated a relatively smooth exterior limiting coat.

The results of experiments designed to follow the uptake of marker substances, in addition to virus particles, by the cells in tissue culture substantiate the previous observations made on the Rauscher virus propagated *in vitro*. The infected cells grown in 4× BME, with ferritin added, exhibited large inclusion bodies containing both degenerating viral particles and masses of ferritin (figs. 16, 17, and 18). Inclusion bodies without ferritin were also observed (fig. 16). The ferritin, debris, and virus particles were contained in membrane-enclosed vacuoles. We have attemped to arrange several micrographs in what is thought to be the proper sequence to illustrate the infected cells in the process of phagocytosing colloidal gold. The infected cells released virus by the budding process (figs. 19 and 20). The gold particles were first observed outside the cells or associated with the plasma membrane (fig. 19). The gold and viral particles then appeared in intracellular vacuoles of relatively low electron density (figs. 20 and 21). The gold particles were also found deeper in the cytoplasm where the density of the vacuolar content is greater, and the viral particles are still in evidence. Cells with many inclusion bodies frequently showed signs of cytoplasmic degeneration (fig. 22). This correlates with observations by light microscopy on the infected cultures where cell senescence, death, and lysis become readily apparent. The background matrix in the cytoplasm appeared condensed or was absent. Profiles of the endoplasmic reticulum were circular (microsomal) rather than flattened cisternae. Gold and viral particles were observed within these degenerating cells. At somewhat higher magnification, the viral particles contained by the phagosomes within degenerating cells frequently appeared reasonably intact (figs. 23, 24, and 25). The particles were comprised of a head region and, occasionally, an elongate tail.

DISCUSSION

Electron microscopic confirmation of the replication of Rauscher virus in tissue culture has been obtained. The extensive number of particles observed correlate with infectivity studies on concentrated viral preparations of supernatants from these cultures (*14*, *15*). There are obvious morphological differences between the Rauscher-virus-infected cells grown in NCTC 109 and in 4× BME medium. Whether or not the virus-cell relationship is primarily responsible for the transformation observed in the 4× BME cultures has not been resolved. However, the association of the virus with the cell membranes, plus the change of media to 4× BME is most probably intimately associated with the induction of the cellular changes. The morphological evidence indicated that there was an increase in cytoplasmic vesiculation and vacuolation. Virus particles were associated with the peripheral plasma membrane and the extensive membrane system in the vesiculated cells, as well as within the membrane-bound phagosomes.

Observations by Fink on the location of fluorescent-labeled anti-Rauscher sera suggest that intracytoplasmic bodies, presumably phagosomes, are frequently the most brilliantly stained (*24*). These morphological studies confirm the immunological studies in that the intracytoplasmic bodies, which are undoubtedly phagosomes, contain viral particles.

Phagocytosis of virus by infected cells that proliferate viral particles has been established by these studies. This observation suggests that possibly the complete viral cycle may exist with only a single cell involved. The cell becomes infected and new virus is replicated and released which, in turn, may be phagocytosed by the same cell, presumably as a digestive process or as an alternative, may infect another cell. It is assumed in this case, as with other viruses, that the process described herein as phagocytosis is not the infective process *per se*. The infective process of the murine viruses has probably not been demonstrated. The observation of virus budding into the intracytoplasmic membrane-lined vesicles suggests that the virus buds from suitable membrane systems wherever they occur. In studies thus far, "C"-type particles bud only from membranes morphologically resembling limiting plasma membranes; *i.e.*, membranes that readily exhibit the "unit" or "leaflet-layered" structure characteristic of the plasmalemma (*25*). "C"-type buds have not been associated with membranes obviously continuous with the ergastoplasm, nuclear membranes, mitochrondrial or Golgi membranes. These observations suggest that the the budding forms which were seen deep in the cytoplasm are proliferating directly into phagosomes, rather than being proliferated from the periphery of the cell and then phagocytosed. In effect, they are short-circuited to destruction or are "born unto death" without the opportunity of infecting a different cell. There is no evidence that the viral particles observed in these regions are released to the extracellular environment. This concept of the phagosome is in modification of hypotheses perpetuated by Beard and collaborators concerning the "gray bodies" and the association with avian leukosis virus (*26*). These authors originally believed that the "gray bodies" were the specific site of viral synthesis. Heine *et al.* reversed this impression by demonstrating that the "gray bodies" arose as a result of phagocytosis (*20*). Our studies suggest that the "gray bodies" or phagosomes may represent both, *i.e.*, they are primarily the result of phagocytosis, but the viral moiety within the cell cannot distinguish between these membrane-bound regions from the peripheral plasma membrane and occasionally bud directly into them.

These studies provide significant evidence that vast quantities of virus are phagocytized by the same cells capable of proliferating the virus. Because large numbers of the particles appear to be intimately associated with the plasma membrane, and the phagosomes containing virus are evident directly below the surface of the cell, probably only a relatively small proportion of the mature particles is permanently released into the tissue culture fluid. It is suggested that a state of dynamic equilibrium occurs with respect to virus titer in the supernatant fluid; therefore, critical assay of these titers may not reflect the total capability of these cultures with regard to viral production. Procedures designed to inhibit the phagocytic activity of these cells without affecting their viral production capacity could conceivably yield vastly greater quantities of infectious virus particles.

The replication of the Rauscher virus in tissue culture afforded ample opportunity to examine the budding phenomenon in thin section. The observation of particles with twin nucleoids suggests that the mechanisms for packaging nucleoid material are precise with respect to quantitation. Otherwise, one might expect giant single nucleoids of 2 or 3 times the normal nucleoprotein complex to be present.

From the comparison of well-resolved, thin-sectioned particles with the negatively stained particles from density gradient preparations, it is apparent that the Rauscher virus is not composed of capsomeric subunits arranged in cubic symmetry. In addition, the strands of ±30 A subunits appear less complex than the helical symmetry exemplified by the myxoviruses or paramyxoviruses, *i.e.*, influenza or mumps viruses (*27*, *28*). The immature particles afforded the best opportunity for observation and analysis of unit arrangement in the nucleoid region. The intermediate shell and the slightly more dense inner shell are formed simultaneously as if by addition of units at their edges. The nucleoid shells are apparently completed by the time the particle separates from the cell, but the final maturation process, which involves the appearance of dense material within the central nucleoid region, apparently occurs after the particle becomes separated from the cell. Since several oncogenic viruses replicate in cells that remain unaltered, it would indeed be interesting to know whether the intracellular environment is protected from active viral nucleic acid during the reproduction stages and whether the maturation stages of the virus particles, reflected by the increase in the metalophilia of the nucleoid, indicate a change in the nucleoprotein from an "unexposed" to an "exposed" state.

Electron-micrographed instances of the Rauscher virus budding from cytoplasmic extensions, plus the observation of tailed particles *in vitro* as well as *in vivo*, lend impetus to the hypothesis that the tail-like structures are derived from excess limiting membrane at the time of budding, and are not pure artifacts. That the particles are susceptible to osmotic as well as other physical effects (*29*) was previously demonstrated (*13*).

The studies presented here on the morphology and the propagation of the Rauscher virus *in vitro* confirm and extend the observations on the sites and mode of the formation of this murine virus. The tissue culture system will prove even more useful in future correlated morphological and biological studies on the infective process of the agents.

SUMMARY

Electron microscopic observations on a murine leukemia virus (Rauscher) propagated in tissue culture have confirmed and extended previous studies. The cytoplasm of cells subcultured in Eagle's basic medium exhibited morphological changes including extensive vacuolation. The virus particles appeared to be membrane-associated. Budding of virus particles was occasionally observed from the plasmalemma of the cell and from membrane-lined areas apparently within the cells. Comparison of immature virus particles from tissue culture with those from density gradient preparations revealed strong evidence for the determination of internal structure of the virus. Many intracytoplasmic inclusion bodies containing virus particles and other cellular debris were observed. Addition of electron-dense markers (ferritin and colloidal gold) resulted in the appearance of these dense markers plus the virus within the phagosomes. Large quantities of viral particles were phagocytosed by the same cells within which the virus can replicate. The titer of virus acquired from the supernatant of these cultures may not reveal an accurate estimate of the viral-genesis potential of the cultures.

REFERENCES

(*1*) DE HARVEN, E., and FRIEND, C.: Electron microscope study of a cell-free induced leukemia of the mouse: A preliminary report. J Biophys Biochem Cytol 4: 151–156, 1958.

(*2*) ———: Electron microscopy of Swiss mouse leukemia virus. Nat Cancer Inst Monogr 4: 291–311, 1960.

(*2*) ———: Electron microscopy of Swiss mouse leukemia virus. Nat Cancer Inst kemia: A review. *In* Tumors Induced by Viruses: Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York & London, Academic Press Inc., 1962, pp 183–206.

(*4*) DALTON, A. J., LAW, L. W., MOLONEY, J. B., and MANAKER, R. A.: An electron microscopic study of a series of murine lymphoid neoplasms. J Nat Cancer Inst 27: 747–791, 1961.

(*5*) DALTON, A. J.: Micromorphology of murine tumor viruses and of affected cells. Fed Proc 21: 936–941, 1962.

(*6*) ———: The Moloney agent. *In* Tumors Induced by Viruses; Ultrastructural Studies (Dalton, A. J., and Haguenau, F., eds.). New York & London, Academic Press Inc., 1962, pp 207–218.

(*7*) DMOCHOWSKI, L., GROSS, L., and PADGETT, F.: Electron microscopic studies of rat leukemia induced with mouse leukemia virus. Proc Soc Exp Biol Med 110: 504–508, 1962.

(*8*) FELDMAN, D. G., GROSS, L., and DREYFUSS, Y.: Electron microscopic study of the passage A mouse leukemia virus in mammary glands of pregnant, virus-injected, C3H(f) mice. Cancer Res 23: 1604–1607, 1963.

(*9*) DALTON, A. J., MOLONEY, J. B., PORTER, G. H., FREI, E., and MITCHELL, E.: Studies on murine and human leukemia. Trans Assoc Amer Physicians 77: 52–63, 1964.

(*10*) DMOCHOWSKI, L., PADGETT, F., and GROSS, L.: An electron microscope study of cat leukemia induced with mouse leukemia virus (Gross). Cancer Res 24: 869–899, 1964.

(*11*) FELDMAN, D. G., and GROSS, L.: Electron-microscopic study of the mouse leukemia virus (Gross), and of tissues from mice with virus-induced leukemia. Cancer Res 24: 1760–1783, 1964.

(*12*) DALTON, A. J., and MOLONEY, J. B.: Recovery of virus from the blood of rats with induced leukemia. *In* The Interpretation of Ultrastructure. Symposia of the Intern Soc for Cell Biol. New York & London, Academic Press Inc., vol. 1, 1962, pp 385–392.

(*13*) ZEIGEL, R. F., and RAUSCHER, F. J.: Electron microscopic and bioassay studies on a murine leukemia virus (Rauscher). I. Effects of physicochemical treatments on the morphology and biological activity of the virus. J Nat Cancer Inst 32: 1277–1307, 1964.

(*14*) WRIGHT, B. S., and LASFARGUES, J. C.: Long-term propagation of the Rauscher murine leukemia virus in tissue culture. J Nat Cancer Inst 35: 319–327, 1965.

(*15*) TYNDALL, R. L., VIDRINE, J. G., TEETER, E., UPTON, A. C., HARRIS, W. W., and FINK, M. A.: Cytopathogenic effects in a cell culture infected with a murine leukemia virus. Proc Soc Biol Med 119: 186–189, 1965.

(*16*) DALTON, A. J.: A chrome-osmium fixative for electron microscopy. Anat Rec 121: 281, 1955.

(*17*) ZEIGEL, R. F., and DALTON, A. J.: Speculations based on the morphology of the Golgi systems in several types of protein-secreting cells. J Cell Biol 15: 45–54, 1962.

(*18*) MOLLENHAUER, H. H.: Plastic embedding mixtures for use in electron microscopy. Stain Techn 39: 111–114, 1964.

(*19*) REYNOLDS, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212, 1963.

(*20*) HEINE, U., BONAR, R. A., BECKER, C., and BEARD, J. W.: Lysosome system of avian leukemic myeloblasts. Nat Cancer Inst Monogr 17: 677–699, 1964.

(*21*) O'CONNOR, T. E., RAUSCHER, F. J., and ZEIGEL, R. F.: Density gradient centrifugation of a murine leukemia virus. Science 144: 1144–1147, 1964.

(*22*) BRENNER, S., and HORNE, R. W.: A negative staining technique for high resolution electron microscopy of viruses. Biochim Biophys Acta 34: 103–110, 1959.

(*23*) ZEIGEL, R. F., and RAUSCHER, F. J.: Electron microscopic and bioassay studies on a murine leukemia virus (Rauscher): Preliminary report. J Nat Cancer Inst 30: 207–219, 1963.

(24) FINK, M. A., and MALMGREN, R. A.: Fluorescent antibody studies of the viral antigen in a murine leukemia (Rauscher). J Nat Cancer Inst 31: 1111–1121, 1963.

(25) ROBERTSON, J. D.: The unit membrane. *In* Electron Microscopy in Anatomy (Boyd, J. D., Johnson, F. R., and Lever, J. D., eds.). Baltimore, Williams & Wilkins, 1961, pp 74–99.

(26) BONAR, R. A., WEINSTEIN, D., SOMMER, J. R., BEARD, D., and BEARD, J. W.: Virus of avian myeloblastosis. XVII. Morphology of progressive virus-myeloblast interactions *in vitro*. Nat Cancer Inst Monogr 4: 251–290, 1960.

(27) HORNE, R. W., WATERSON, A. P., WILDY, P., and FARNHAM, A. E.: The structure and composition of the myxoviruses. Virology 11: 79–98, 1960.

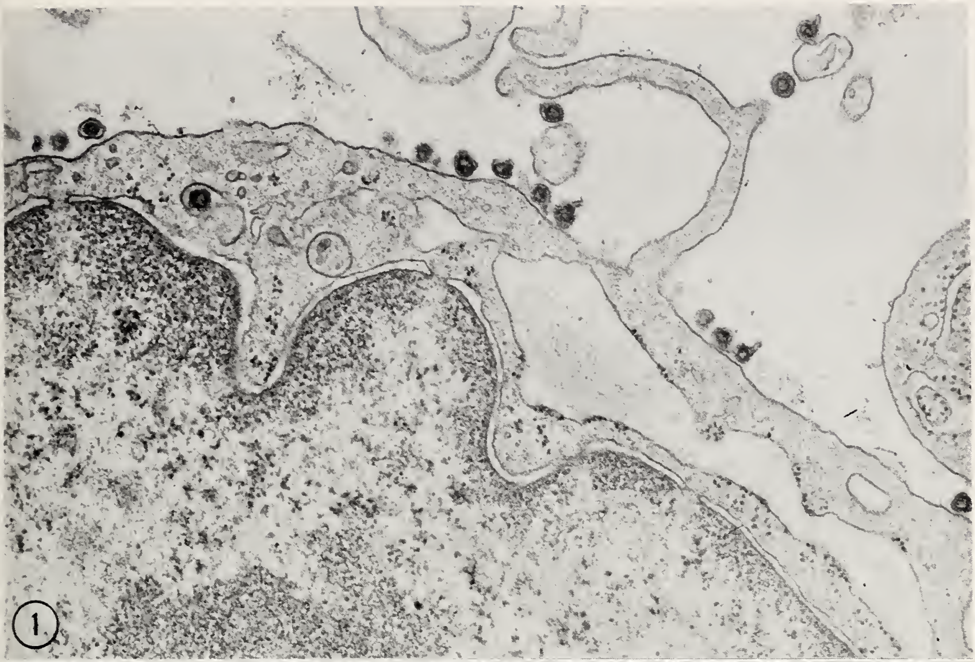
(28) HORNE, R. W., and WATERSON, A. P.: A helical structure in mumps, Newcastle disease and Sendai viruses. J Molec Biol 2: 75–77, 1960.

(29) LEVY, J. P., BOIRON, M., SILVESTRE, D., and BERNARD, J.: The ultrastructure of Rauscher virus. Virology 26: 146–150, 1965.

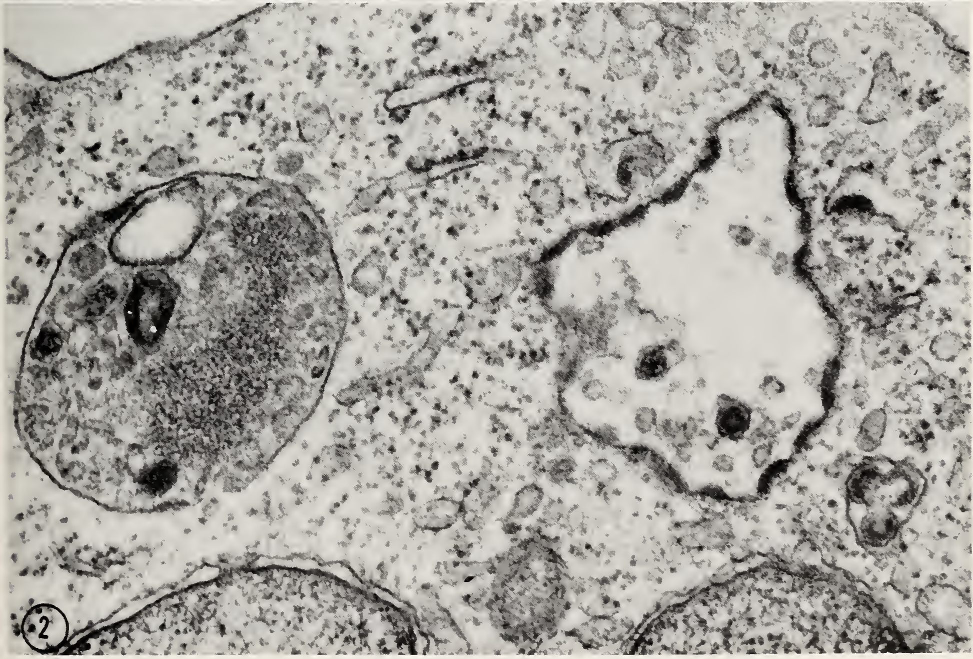
Plate 65

Figure 1.—Cells infected with Rauscher virus and grown in NCTC 109 medium exhibit relatively normal-appearing nuclear and cytoplasmic characteristics. Occasionally, the cisternae of the endoplasmic reticulum are dilated. Viral particles appear in intimate association with the plasmalemma. × approximately 30,000

Figure 2.—Rauscher-virus-infected cells grown in NCTC 109 medium occasionally had particles budding into membrane-lined vessicles within the cells. It is thought that these areas represent pre-lysosomal areas. Typical phagosomes are also visible. × approximately 52,500



①

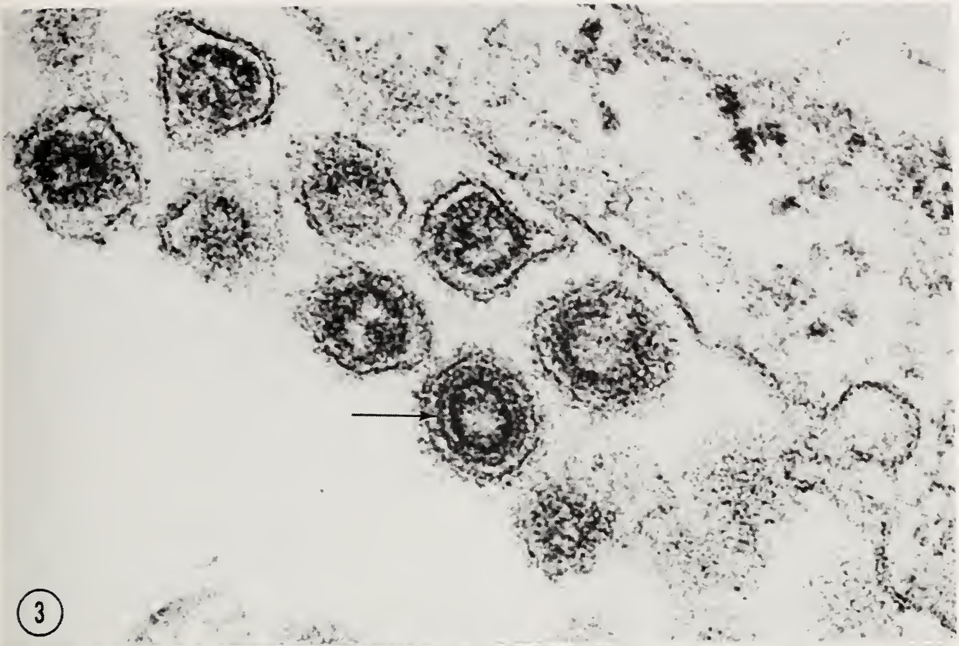


②

PLATE 66

FIGURE 3.—Immature and mature viral particles are similar to those described *in vivo*. The intermediate ring is apparent in the immature particles (*arrow*) which were observed in cultures grown in NCTC 109 medium. × approximately 171,000

FIGURE 4.—Particles with double nucleoids were occasionally observed. "Twin" particles were also seen in negatively stained preparations of concentrated blood plasma extracts. These particles were observed in cultures grown in NCTC 109 medium. × approximately 150,000


3

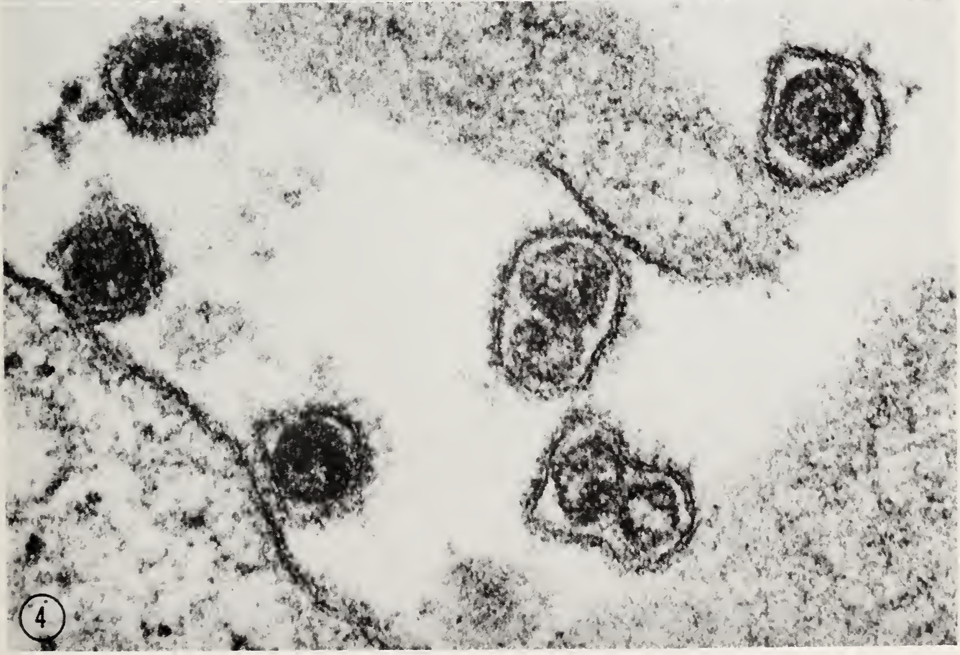
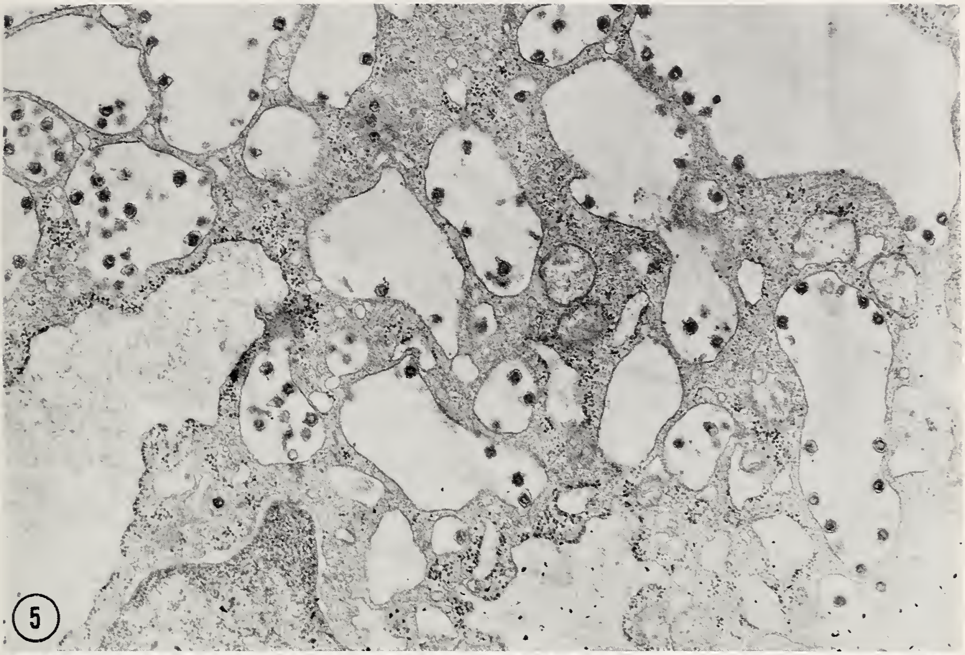

4

PLATE 67

FIGURE 5.—Rauscher-virus-infected cells grown in 4× BME frequently exhibit vacuolated cytoplasm. Most of these membrane-lined areas do not have ribosomes associated with their cytoplasmic faces. They appear similar to the plasmalemma. It is suggested that these areas are true, unconnected vacuoles and not environment-connected channels. Many viral particles are associated with the membranes. × approximately 20,000

FIGURE 6.—Cells grown in 4× BME also contain many membrane-bound bodies with membranous debris and viral particles. These are apparently the result of phagocytosis. Virus particles are also apparent in the extracellular spaces. × approximately 20,000


5

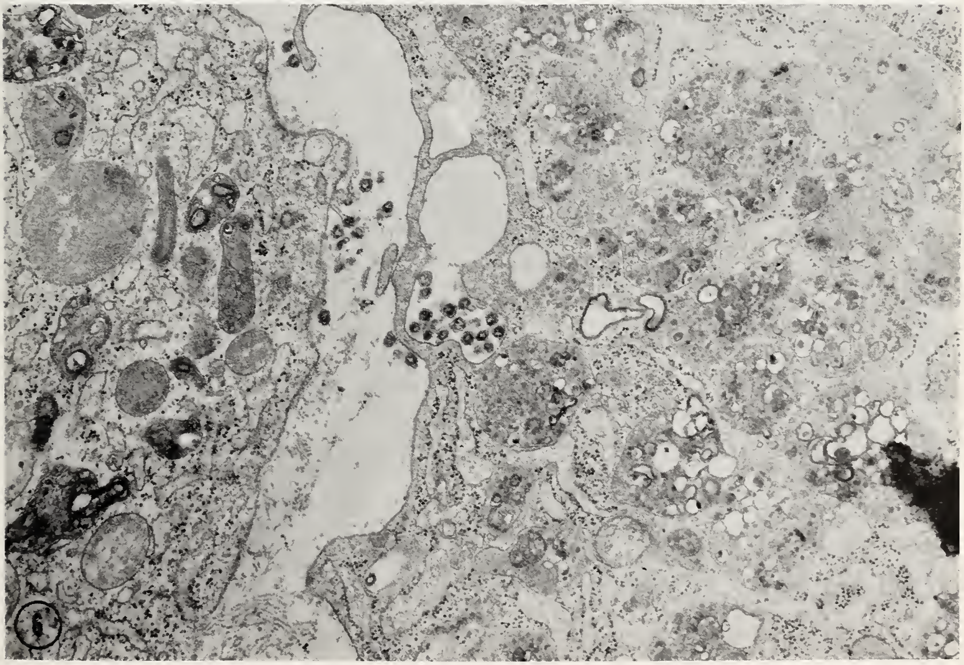
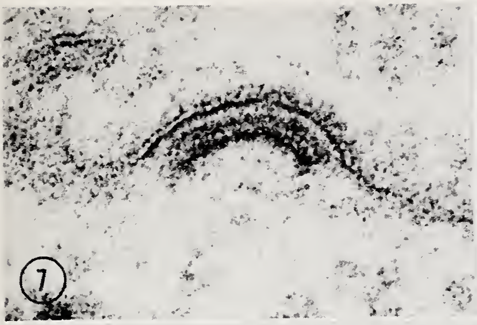
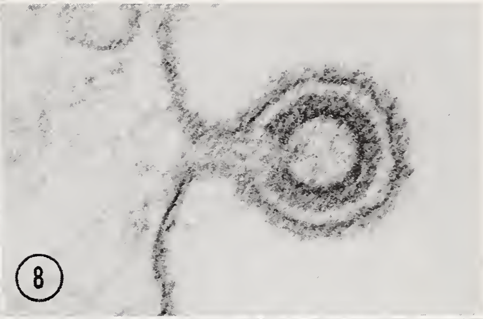
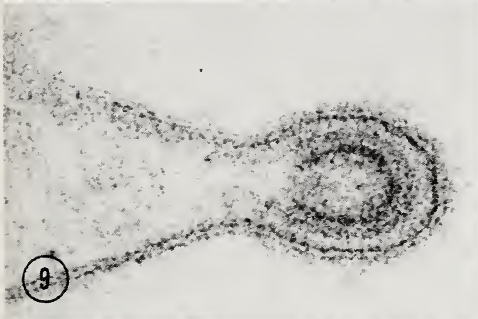

6

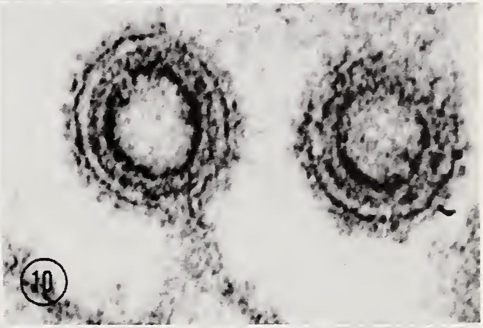
PLATE 68

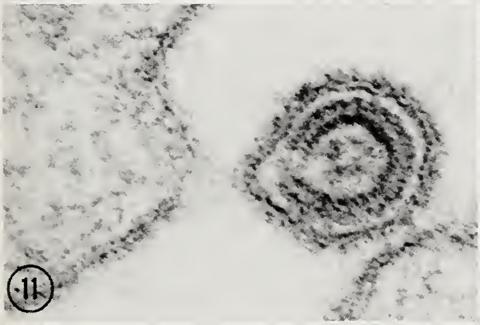
FIGURES 7 THROUGH 14.—These figures demonstrate the Rauscher virus in various stages of budding from the plasma membranes. They demonstrate the inner and intermediate shells as they form as a crescent. Outer coat is seen to be continuous with the plasmalemma of the cell. They occasionally bud from cytoplasmic stalks. It has been suggested (*13*) that this process may account for the pleomorphic tail-like extensions observed in thin-sectioned and negatively stained preparations. × approximately 225,000

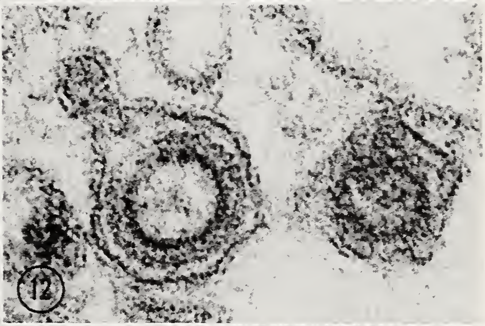

⑦

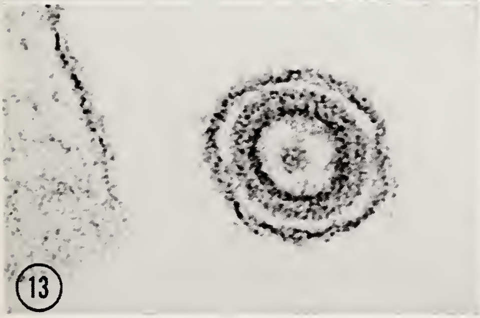

⑧


⑨


⑩


⑪


⑫


⑬

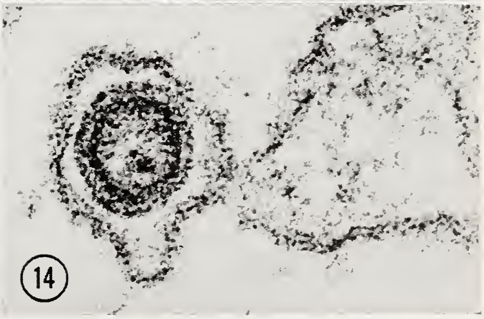
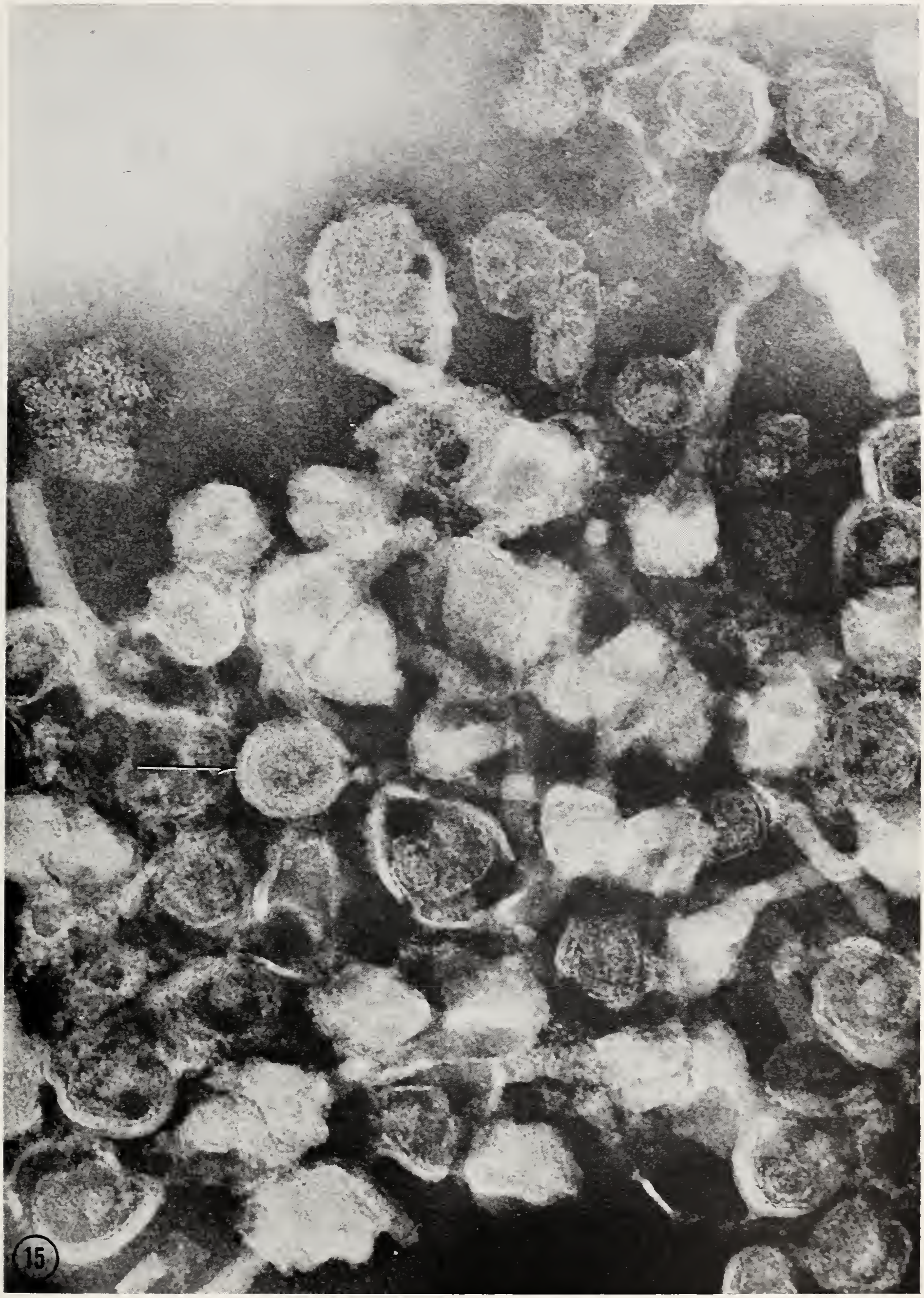

⑭

Plate 69

Figure 15.—Negatively stained (PTA) Rauscher virus, prepared from a cesium chloride density gradient fraction reveals the fine structure of the particles against a relatively low-noise background. The coat material is occasionally partially removed from the nucleoid region, which appears to be composed of a relatively simple meshwork of strands of small (±30 A) subunits. It is not known whether these strands are helical. Several of the particles possess tails. A particle interpreted as an immature particle is illustrated (*arrow*). × approximately 160,000



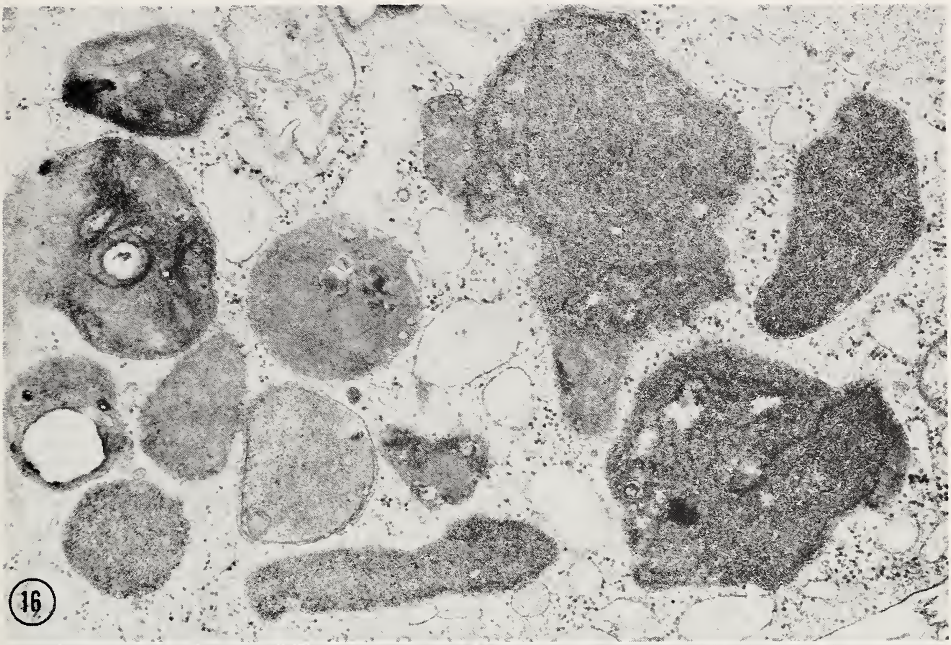
15

PLATE 70

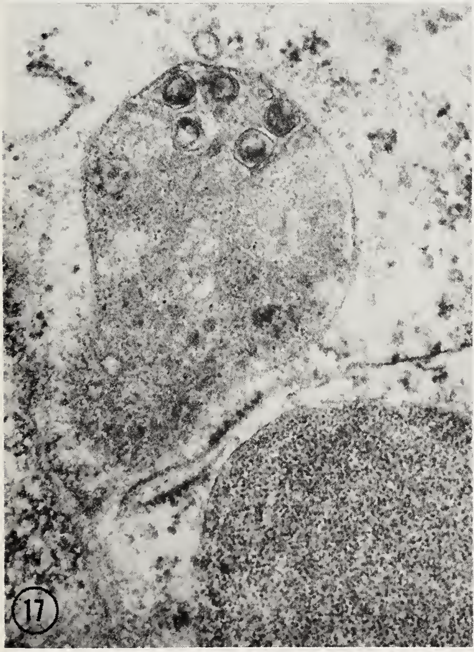
FIGURE 16.—Rauscher-virus-infected cultures grown in 4× BME medium and inoculated with unpurified ferritin possess phagosomes containing masses of ferritin. Phagosomes without ferritin are also present. The phagosomes also contain mature viral particles. × approximately 30,000

FIGURE 17.—Intracytoplasmic phagosomes contain both ferritin and virus particles. Occasionally, the ferritin is not highly concentrated. × approximately 54,000

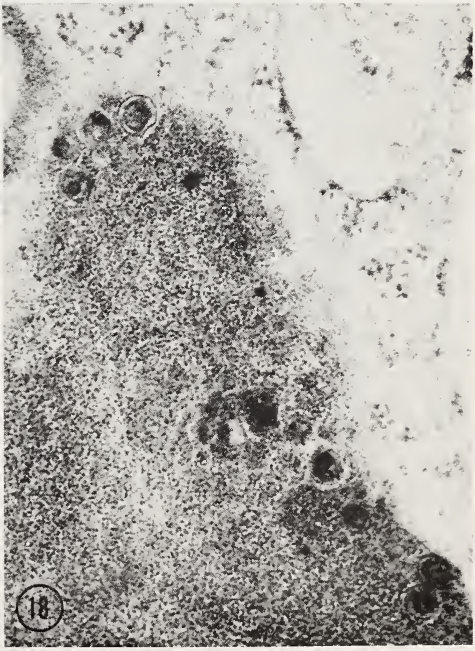
FIGURE 18.—A phagosome containing both ferritin and viral particles is demonstrated. The intracellular bodies probably represent the result of active phagocytosis of extracellular material. × approximately 54,000



⑯



⑰

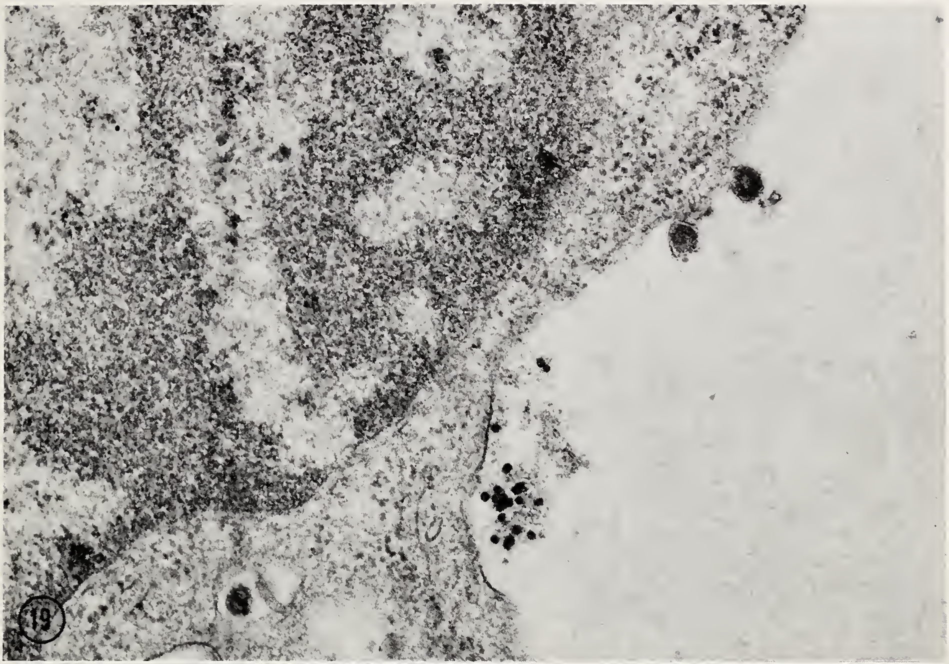


⑱

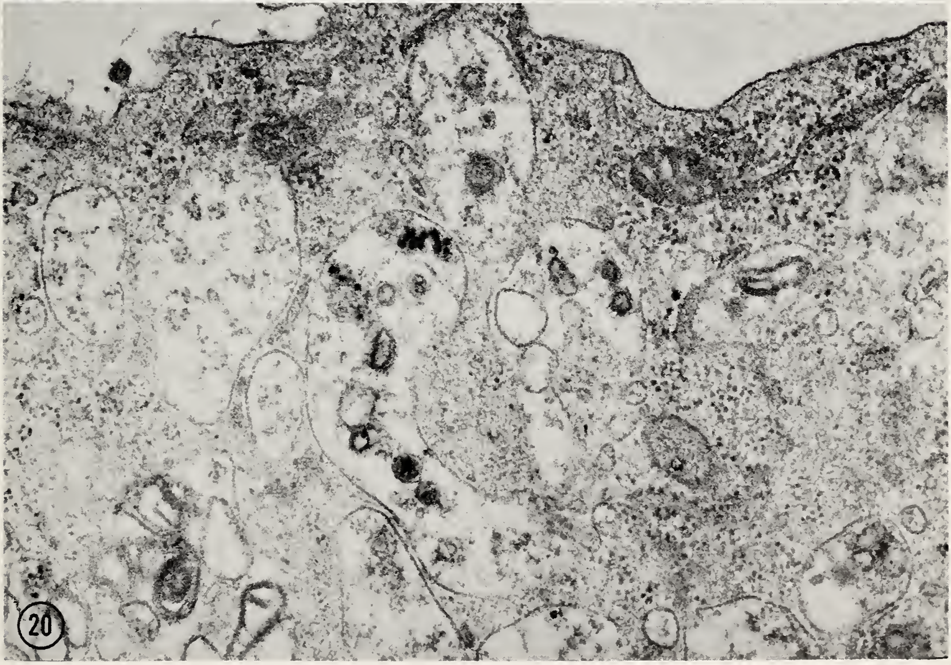
Plate 71

Figure 19.—Portion of a cell from a culture grown in 4× BME and inoculated with colloidal gold. A viral bud is originating from the plasmalemma. The dense, gold particles are membrane-associated. × approximately 54,000

Figure 20.—A cell, which is apparently phagocytosing virus and colloidal gold, also has a viral bud at its surface. The phagosome matrix is relatively electron lucent and is suspected of being relatively young in comparison with the dense phagosomes observed deeper in the cytoplasm. × approximately 45,000



⑲

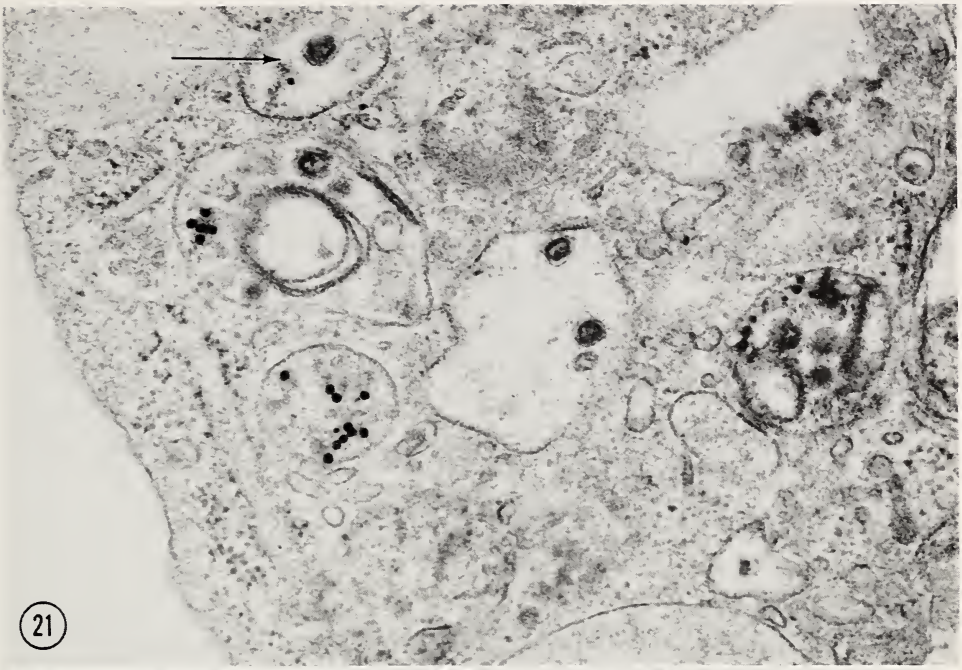


⑳

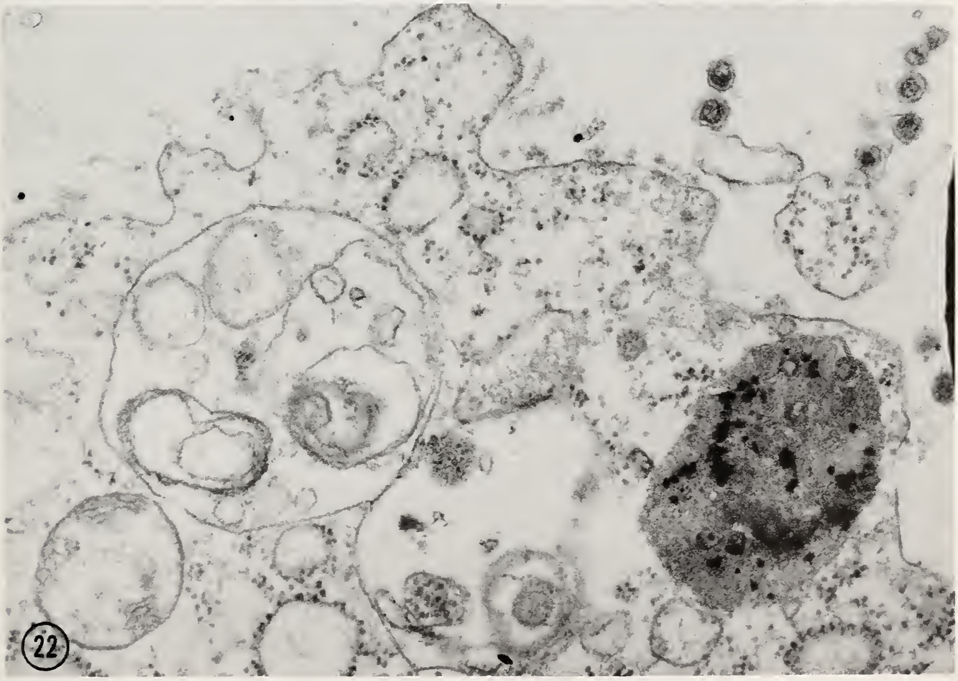
PLATE 72

FIGURE 21.—Portion of a cell from a Rauscher-virus-infected culture grown in 4× BME and inoculated with colloidal gold exhibits several phagosomes containing both viral particles and gold particles. Only one gold or virus particle need be present to be recognized (*arrow*). × approximately 54,000

FIGURE 22.—A cell from a culture infected with Rauscher virus, grown in 4× BME and inoculated with colloidal gold, exhibits signs of degeneration. A "mature" phagosome with dense matrix and myelin-like membrane elements is visible. Viral particles and colloidal gold are still distinguishable. × approximately 45,000



㉑

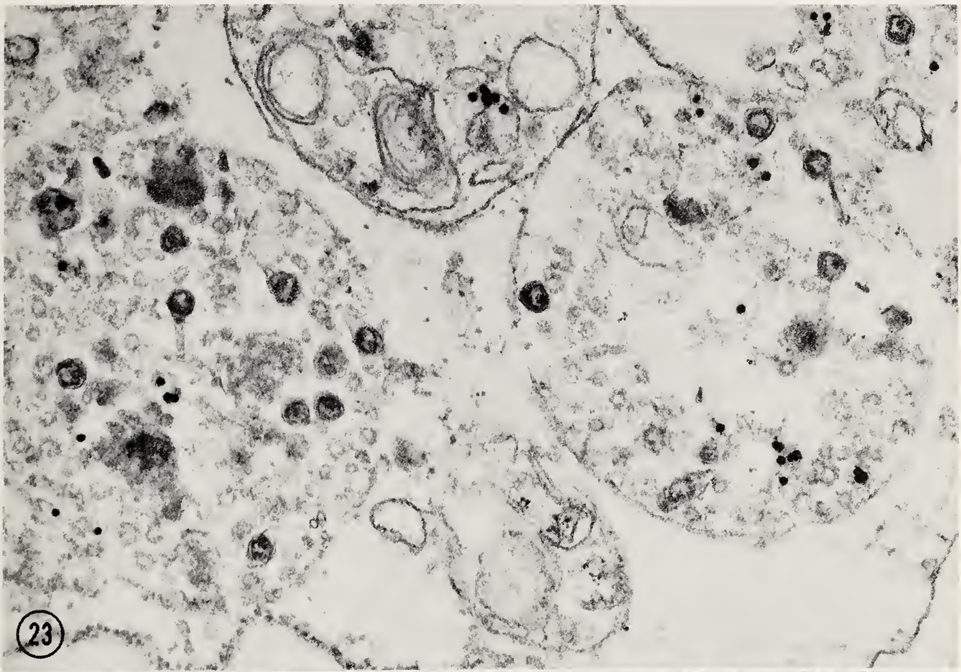


㉒

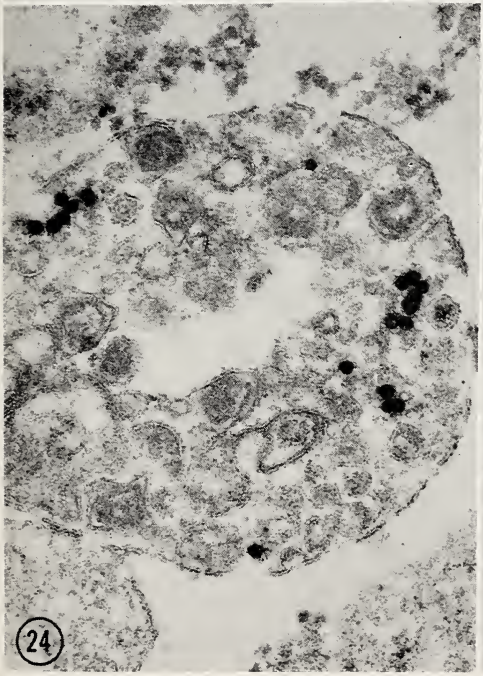
PLATE 73

FIGURE 23.—Phagosomes in degenerating cells contain both gold and virus particles. Tail-like extensions are associated with several of the particles. × approximately 54,000

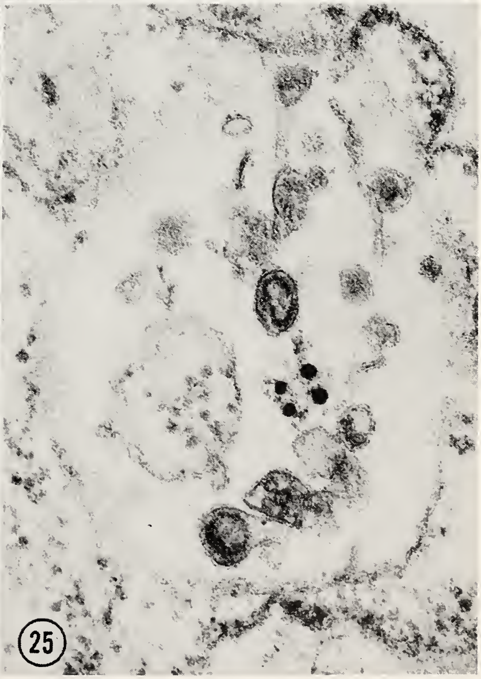
FIGURES 24 AND 25.—At slightly higher magnification, the viral particles are readily resolved. They occasionally possess tail-like extensions. The gold particles also indicate the focal qualities of micrographs. × approximately 90,000



㉓



㉔



㉕

VIRAL, CHEMICAL, AND RADIATION-INDUCED MURINE LEUKEMIA

Chairman: Joseph W. Beard

Transmission Studies of a Leukemogenic Virus, MLV, in Mice[1]

L. W. Law, *Laboratory of Biology, National Cancer Institute,*[2] *Bethesda, Maryland*

THE results reported here are extensions of a study designed to gain some information on the epidemiologic behavior of a leukemogenic virus, Moloney leukemia virus (MLV), in mice.

The study concerns congenital transmission, that is, from parents to offspring, and attempts also to detect transmission of MLV infection horizontally, that is, by contact, possibly through an airborne route or by other means.

Despite the fact that several leukemogenic viruses have now been isolated and concentrated from murine neoplasms, few experimental attempts to induce transmission have been reported. Studies with these viruses are relatively difficult presently, since the sensitive and reliable tools developed for the detection of polyoma virus (*1*), the avian leukosis virus, RIF (*2*), and other tumor viruses are not applicable. The recent development of a rapid, sensitive, and reproducible CF test for laboratory-adapted strains of some murine leukemogenic viruses (*3*) may indeed lend itself to more definitive epidemiologic studies.

CONGENITAL TRANSMISSION

A standardized and virulent preparation of MLV, designated LT (V) 96, was used for all the studies reported here and has been described (*4*). Litters from 1 to 7 days of age were inoculated subcutaneously with 0.1 ml of a 10^{-1} dilution of the MLV preparation. These mice remained with their mothers to weaning at 4 weeks of age. These litters receiving virus parentally are referred to as G0. Successive litters of noninoculated mice were then obtained by matings of siblings and are referred to as

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Table 1.—Induction of leukemia in inbred strains of mice by MLV: studies of transmission to offspring

Strain	% leukemia in recipients (G0)	Maximum No. of generations	% leukemia in offspring	Number of conversions (high leukemic to low leukemic)
C3Hf/Lw	100	13	249/325 (77%)	3 (G4, G7, G8)
C3Hf/Bi	92	15	193/219 (88%)	3 (G3, G4, G8)
C3Hf/Fg [1]	70	14	96/131 (73%)	1 (G8)
RF	90	13	127/157 (81%)	2 (G2, G7)
BALB/c	100	5	152/195 (70%)	None
C58 [2]	10	None with leukemia at <7 months after G0		
C57BL/Ka	20	3	0/43	
AKR	72	None with leukemia at <6 months after G0		
NH	0	Negative leukemia through 3 generations		

[1] The C3Hf/Fg strain is a high-leukemic strain (*5*), so that susceptibility to MLV was measured by the frequency of leukemia occurring before 9 months of age, the age at which spontaneous leukemias first occur in this strain.

[2] Susceptibility to MLV in the AKR and C58 high-leukemic strains was determined by the frequencies before 6 months of age in the recipients.

G1, G2, etc. Matings of siblings were made at random at weaning so that there was no conscious selection of leukemic strains over nonleukemic strains.

The response of several strains of mice to MLV is shown in table 1. Most strains were highly susceptible. The high-leukemic strain C58, C57BL/Ka, and the NH strain were relatively resistant to the leukemogenic activity of MLV. No leukemias were observed in NH mice over a 12-month period. Transmissibility of virus as detected in successive generations (G1, G2, etc.) of inbreeding was studied in all strains. Leukemia was transmitted with relative ease through 13 to 15 inbred generations of 3 C3H sublines and the RF strain and in BALB/c mice studied through only 5 inbreeding generations. On the other hand, AKR mice which were susceptible to MLV did not apparently transmit virus effectively to their offspring. Their behavior was similar to that of the resistant strains C58, C57BL, and NH (table 1). RF mice showed a dichotomy of behavior in their ability to transmit MLV effectively. Although MLV was highly pathogenic for RF mice, some females failed to transmit virus to their offspring, as determined by the appearance of leukemia in offspring. Only effective transmitters are discussed in this report.

These results show that certain strains of mice susceptible to MLV, such as C3Hf/Lw, C3Hf/Bi, C3Hf/Fg, RF, and BALB/c, can effectively transmit virus usually without loss of virulence to succeeding generations of offspring. The results in terms of frequency of leukemia and mean age at death are given for successive inbred generations in table 2. High-leukemic substrains of mice may therefore be established simply through continued inbreeding. It should be pointed out here that most reticular neoplasms observed in MLV-infected mice were of the lymphocytic or stem cell form. For example, the morphologic classification of 81 leukemias studied in the C3Hf*/Bi substrain was as follows: 71 lymphocytic or stem cell, 4 reticulum cell sarcomas, and 6 granulocytic leukemias.

PATTERN OF CONGENITAL TRANSMISSION OF MLV

In order to determine the route of transmission of virus from mother to offspring, reciprocal foster nursings were carried out between the strains: the artificially established high-leukemic substrains and their low-leukemic counterparts. Transmission could occur before birth through placental and extraembryonic membranes to the embryos, after birth through the mother's milk, or both before and after birth. Another possible route of viral transmission congenitally is through the paternal line, either by sperm which conceivably could infect the mother (*6*) or by the spermatozoa at fertilization. This possibility is considered later.

RECIPROCAL FOSTER NURSINGS

Litters born of virus-infected mothers of the several laboratory-established, high-leukemic strains designated RF*, C3H*, etc., were fostered upon low-leukemic mothers of several strains and *vice versa.* Entire litters were foster-nursed. Litters delivered from mothers of early generations (G0 and G1) and those delivered from mothers in late (G7 and G8) inbred generations of the "high-leukemic" strains were used. Special care was taken that the litters delivered from MLV-infected mothers did not obtain milk from their own mothers, through the use of a special cage previously described (*7*). The efficacy of this method of foster nursing has been demonstrated in studies of the transfer of antibodies to polyoma virus (*8*).

It is clear from the combined results of reciprocal foster-nursing experiments (table 3) that transmission of MLV in the prenatal period is very ineffective, at least as recorded by the frequency of lymphocytic neoplasms (groups A, B, and C) and that transmission through mother's milk is highly effective (group D). In those litters from high-leukemic mothers, leukemia was observed in only 5 of 56 litters. Two of these litters were delivered from mothers already exhibiting signs of leukemia. In each litter of 4 and 5, respectively, 2 leukemic animals were observed. In not a single instance was leukemia observed where C57BL mothers were used as foster nurses.

Several laboratory-established, high-leukemic substrains of C3Hf/Bi and C3Hf/Lw mice were obtained simply by foster nursing of previously unexposed litters on infected mothers (group D) and have persisted as such through 9 to 12 generations of continued inbreeding.

Transmission of Passage A leukemogenic virus by way of the mother's milk was observed by Gross (*9*) to be achieved most effectively if the mother was leukemic or in the preleukemic phase of the disease at the time of suckling a litter. In contrast, MLV was shown in these studies to be effectively transmitted via the mother's milk at any age of the mother

TABLE 2.—Leukemia following progeny inbreeding in artificially established high-leukemic substrains

Strains	Number of substrains		Generation												
			G0[1]	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
							c ↗	2/10 (11)	0/6	0/2	0/7	0/9	0/6	—	
C3Hf*/Bi	3	(a)	5/5 (5½)	7/8 (5)	10/10 (6½)	9/9 (5)	8/9 (6)	9/9 (6)	10/10 (5½)	9/11 (6)	20/22 (5½)	4/5 (4½)	10/13 (5)	6/8 (5)	14/15 (5)
					c ↗	0/6	0/6	0/5	0/5	0/6	0/9				
	2	(b)	3/3 (3½)	5/5 (5½)	20/22 (6)	18/22 (6½)	8/8 (6)	3/3 (4½)	5/5 (5)	9/9 (4½)	6/9 (5) c ↘	3/3 (6½)	6/11 (5)	—	
												0/6	0/6	0/5	—
										c ↗	0/4	0/3	0/8	0/14	—
C3Hf*/Fg	3	(a)	3/4 (4)	2/5 (4)	7/13 (5)	25/27 (6)	3/5 (6½)	8/9 (6)	6/11 (6½)	14/16 (5½)	4/11 (5½)	3/4 (5)	4/4 (5)	17/22 (4½)	—
	3	(a)	3/3 (4)	6/6 (8)	12/13 (5½)	15/18 (7½)	14/15 (7½)	10/12 (8)	17/18 (5½)	10/10 (5½)	4/5 (8)	9/12 (5½)	4/6 (4)	—	
											c ↗	0/2	0/7	0/18	—
C3Hf*/Lw	2	(b)	3/3 (8)	7/7 (8)	10/11 (7)	4/5 (7)	9/12 (6½)	6/8 (6)	6/9 (6)	8/12 (7)	5/10 (7)	12/19 (7½)	10/13 (6)	—	
						c ↗	0/4	1/4 (7)	0/4	0/5	0/5	0/5	0/6	—	
	3	(c)	1/1 (5)	14/16 (6)	12/19 (7)	17/22 (6½)	15/17 (6)	6/11 (7)	7/8 (7)	3/4 (6) c →	0/5	0/4	—		

RF*	2	(a)	2/3 (5½)	8/9 (4½)	7/7 (4)	14/14 (4)	9/10 (3½)	5/6 (5)	12/16 (4) $\overset{c}{\nearrow}$	0/3 13/15 (5½)	0/8 20/26 (5)	0/9 10/18 (4½)	— 6/11 (5)	7/8 (5)	7/7 (5)
	1	(b)	3/3 (2½)	4/4 (4½) $\xrightarrow{c}$	0/3	1/18 (9)	0/18	0/12	0/13	—					

---

[1] The original litters (G0) of each of these substrains received MLV subcutaneously at from 1 to 7 days after birth. Subsequent, successive generations (G1 to G12) represent continued inbreeding of the infected mice; no further virus inoculations were made (*see 4, 6*). Numerator = number of leukemic mice. Denominator = total number of mice in generation. Figures in parentheses = average age at leukemic death in months. An *arrow with a "c"* signifies conversion to a low-leukemic substrain.

Successive generations of control mice were maintained in the same rooms and at the same time; these were derived from low-leukemic littermates of the mothers supplying the experimental mice. The frequency of leukemia in the controls was similar to that reported in these strains (*6*).

The frequency of leukemia in C3Hf*/Fg mice was determined up to the age of 9 months, since beyond this time the spontaneous disease develops (*see 5*).

Table 3.—Lymphocytic neoplasms in potentially high-leukemic litters (HL) foster-nursed on low-leukemic mothers (LL)

Group	Foster nurse	Donor strain	Generations	Number of donor litters	Number leukemic / Number of mice	Fate of donor mothers
	(LL)	(HL)				
A	C57BL	RF*	G1–G7	6	0/37	All 6 leukemic at 3½ to 7 months
B	C3H [1]	C3H*	G1–G8	28	7/125	2 mothers leukemic at delivery (2/4, 2/5)
C	C57BL	C3H*	G4–G8	22	0/81 [2]	Majority leukemic
	(HL)	(LL)				
D	C3H* [1]	C3H	G0–G8	17	68/74 [3]	Mothers nonleukemic

---

[1] C3H and C3H* mice were members of two substrains: C3Hf/Bi and C3Hf/Lw. The C57BL/KaLw strain was used here as foster nurses.

[2] Continued inbreeding of some of these mice did not reveal existence of "latent" virus.

[3] One laboratory-established, "high-leukemic" C3H substrain, developed through foster-nursing of an unexposed litter on a C3Hf*/Lw mother. has continued as a leukemic strain for 9 successive generations (32/37 leukemic).

(table 4). The frequency and age at death from leukemia were similar in the two groups studied: litters born of high-leukemic mothers 6 to 9 weeks of age (first litters) and litters born subsequently (second to fourth litters) when mothers were 10 to 24 weeks of age, some in the preleukemic phase of disease (*cf* group II, table 4). As studied in this laboratory, Passage A virus was highly pathogenic for C3Hf/Bi mice (29/29 leukemic with death at 2½ to 4 months), yet transmission to offspring through the mother's milk was not effective (group II, table 4).

Another factor, revealed by the present studies, which appears not to influence the potentiality of offspring to develop leukemia, is the eventual fate of the mother, that is, whether or not she becomes leukemic. There were 13 mothers representing the G1 to G9 generations from the 3 high-leukemic C3H* substrains which delivered 19 litters. All litters had leukemic offspring (68/99 = 70%), yet all of the mothers remained nonleukemic until death at 9 to 16 months. This frequency is similar to that observed among offspring delivered of mothers which eventually became leukemic.

TRANSMISSION OF MLV BY VIREMIC MALES

To evaluate the role of potentially leukemic males in transmission of virus to their offspring, 16 males from 3 high-leukemic substrains (RF*, C3Hf*/Bi, and C3Hf*/Fg) were mated with females of the corresponding low-leukemic strains, and the resulting progeny was observed for leukemia. The males were selected from the G1 to G8 generations; all eventually became leukemic (*see* table 5). There were 32 litters obtained from these

Table 4.—Frequency of leukemia in first litters compared with subsequent litters from high-leukemic mothers

Group	Strain	First litters (6–9 weeks)			Second, third, and fourth litters (10–24 weeks)		
		Number	Number leukemic / Number of mice	Age leukemic (months)	Number	Number leukemic / Number of mice	Age leukemic (months)
I	RF*	5	13/16 (81. 3%)	4. 2	7	24/33 (73%)	5. 2
	C3Hf*/Lw	8	20/25 (80%)	6. 8	11	34/39 (87. 2%)	6. 9
	C3Hf*/Bi	7	22/33 (66. 7%)	6. 1	7	25/29 (86. 2%)	5. 5
II	C3Hf/Bi (Passage A)	6	3/31 (9. 6%)[1]	4. 8		—	

[1] *See* Gross (*9*) for transmission of virus to offspring from preleukemic or leukemic mothers. All C3Hf/Bi mothers used to obtain offspring here developed leukemic but at 1 to 2 months after delivery of these litters.

Table 5.—Leukemia in offspring of matings: potentially leukemic ♂♂ × normal (low-leukemic) ♀♀

Group	Strain	Number of males used	Fate of males (L = leukemia)	Offspring		Age at leukemic death (months)
				Number of litters	Number leukemic / Number of mice	
A	RF*	5	L3, L5, L5½, L6	11	1/45	5
	C3Hf*/Bi	6	All leukemic at 5½–9 months	9	1/45 [1]	10
	C3Hf*/Fg	5	L4, L4, L4½, L6, L8	12	0/47 [2]	—
B	AKR	Mated with C3H females			48/85 (56. 5%)	14. 4

[1] Seven subsequent litters in G2 and G3 obtained; none were leukemic among 31 mice observed to 12 months of age.

[2] All mice of subsequent generations, G1–G3, were nonleukemic to 9 months of age. Beyond 9 months, spontaneous leukemia appears within this strain. The frequency in this sample (11/47) at 9–16 months of age is similar to controls.

matings; several litters were the second and third litters from a single mating. It may be seen from table 5 that none of the known viremic males effectively transmitted infection to their progeny.

Total-body X irradiation with 300 r in a sample of 22 offspring obtained from matings with RF * males and RF females induced 4 lymphomas, 1 granulocytic leukemia in a 6-month-old male, and 3 thymic lymphosarcomas in females (7, 8, and 6 months old); a similar frequency (3/20 lymphomas) occurred in a 12-month period among irradiated RF, low-leukemic mice. These results indicate that transmission of low concentrations of MLV or of "latent" virus did not occur through the paternal line.

In the fowl, the failure of viremic roosters to transmit RIF infection is known not to be related to inactivation of virus carried in the sperm by antibody present in the oviduct nor to a resistance of testicular cells to viral infection (*2*). The failure of male transmission in the fowl as well as the failure observed here may indeed be related to the loss of virus from spermatozoa as a result of shedding of cytoplasm and the marked loss of RNA, and hence of viral genome, which accompanies spermatogenesis (*10*).

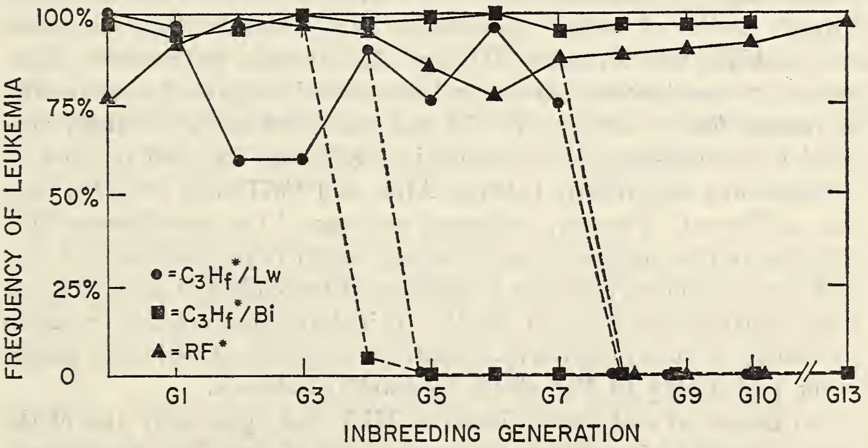
In contrast to the failure of these laboratory-established, high-leukemic males to transmit infection, males of certain spontaneous high-leukemic strains, such as AKR, C58, and C3Hf/Fg, regularly transmit the potentiality to develop leukemia to their progeny (*see* group B, table 5). Reciprocal crosses using these high-leukemic strains mated with low-leukemic strains usually yield similar frequencies of leukemia in the offspring. If it is assumed, therefore, that the virus commonly isolated from the leukemic tissues of AKR mice, for example, is the causative agent for the leukemias occurring regularly within the strain, there must exist a very intimate association between the viral genome and chromosomes of the host cell (germinal as well as somatic) similar to that which occurs in lysogenic bacteria.

SPONTANEOUS CONVERSIONS OF HIGH-LEUKEMIC TO LOW-LEUKEMIC SUBSTRAINS

In nearly 20% of the substrains of high-leukemic (HL) mice which were continuously inbred by brother × sister matings, after parenteral introduction of MLV, a rapid conversion to low-leukemic (LL) strains (nonleukemic) occurred (*see* tables 1 and 2). Text-figure 1 shows the generation in which four typical conversions (HL→LL) occurred and the resulting frequencies of leukemia. In three of these substrains a prior litter was obtained and observation of the offspring in subsequent generations showed these to behave as high-leukemic substrains. A characteristic feature of all conversions, except one, was the rapidity of the conversion. Reciprocal conversions (LL→HL) were never seen among our control mice. Interestingly, these conversions from high-leukemic to low-leukemic substrains were observed in C3Hf */Lw, C3Hf */Bi, C3Hf/Fg, and RF * but not in BALB */c.

The following conclusions appear warranted from the above results and those published previously concerning congenital transmission of MLV:

1) Transmission of virus occurs effectively through the mother's milk. Mothers are viremic and capable of transmitting virus at any age and prior to the development of preleukemic or leukemic lesions. Other viruses are less effectively transmitted congenitally (*see 9, 11, 12*), except the P-LLV virus described by Precerutti and Law (*13*). Some strains of mice, *e.g.*, AKR, C58, C57BL, and NH, do not transmit MLV to their progeny even though the virus may be highly pathogenic in the original recipient.



TEXT-FIGURE 1.—The origin of some low-leukemic substrains by conversion from laboratory-established, high-leukemic substrains (HL→LL). These conversions (*broken lines*) occurred with different frequencies in all established substrains except BALB/c. Conversions from a high to a low substrain were rapid. *See* text for discussion.

2) Effective transmission of MLV across placental membranes occurs rarely under the natural condition studied here. Ida *et al.* (*14*) have reported evidence for the transfer of MLV from strain A and BALB/c mothers during the prenatal period; the mothers used, however, were infected parenterally during the period of pregnancy. Gross (*9*) also obtained a low frequency of leukemia among litters born to virus-infected mice fostered upon virus-free C3H mothers. These mice, however, remained with their virus-infected mothers for varying periods of time and the likelihood that they obtained some of their mother's (virus-infected) milk was recognized by Gross. To obtain unequivocal evidence of passage of MLV across placental membranes, it will be necessary to use the strictest of precautions and resort to cesarean sections in such experiments. Clearly, from the data reported here, however, MLV is not regularly transmitted via germinal cells.

3) High-leukemic substrains of mice may be established simply by parenteral introduction of virus or by foster nursing. This occurs if the strain is susceptible to virus and also capable of replicating and transmitting virus. Low-leukemic substrains may be established by foster nursing, providing the progeny do not receive mother's milk.

4) No evidence was obtained to indicate effective transmission of virus to progeny by infected males.

5) The laboratory-established, "high-leukemic" substrains studied in this laboratory are not completely stable. Spontaneous conversions to "low-leukemic" substrains have occurred. The factors contributing to these conversions have not been identified.

The results reported here contrast sharply with the facts developed through studies of certain spontaneous high-leukemic strains of mice, such as AKR, C58, F, and C3Hf/Fg. All attempts to transform high-leukemic to low-leukemic strains and *vice versa* have proved unsuccessful. Reciprocal foster nursings (*15–17*) and successive foster nursings, combined with successive thymectomies in AKR mice (*6*) and transfer of fertilized ova reciprocally between AKR and C3H mice (*18*, *19*), have been ineffectual. Further, reciprocal matings of the spontaneous high-leukemic and low-leukemic strains usually result in populations of F_1 hybrid mice having very similar frequencies of leukemia and average age at death from the disease (*15–17*, *20*, *21*). It is clear, therefore, that in certain high-leukemic substrains the male contributes equally as well as the female to the potentiality of F_1 hybrids to develop leukemia.

The failure of male transmission of MLV and apparently also of the Gross Passage A virus may be the result of loss of virus from spermatozoa with the shedding of cytoplasm, as suggested for an avian leukosis virus (*2*). The precise and rapid *in vitro* (*3*) bioassay techniques now available for these viruses may resolve this question.

ATTEMPTS AT TRANSMISSION THROUGH CONTACT INFECTION

In these studies, experiments were designed to detect transmission of MLV from experimentally infected mice to susceptible mice of several strains by contact infection at various ages. In addition, infectivity of oral washings and urine was studied in the naturally infected mice at various times after MLV infection was transmitted through mother's milk.

MLV-infected mice were from the high-leukemic substrains described above (*see* table 2). Generations G4 to G10, after introduction of virus, were used either as donors for urine and oral washings or as *infector* mice in the contact experiments. Noninfected but susceptible mice of the inbred strains BALB/cLw, C3Hf/Lw, C3Hf/Bi, and RF were used either as recipients of parenteral infections or as *contact* mice. These were obtained from our inbred colonies.

Potentially leukemic litters (*infector* mice) of C3Hf*/Lw and C3Hf*/-Bi were placed in contact with susceptible but uninfected litters of BALB/c, C3Hf/Lw, or C3Hf/Bi mice. The *infector* mice were from the G4 to G8 generations of high-leukemic strains and had obtained virus through the mother's milk. The particular families selected were those with a history of more than 80% leukemia (table 2). *Infector* mice were removed from their mothers and placed with the *contact* mice when 3 to 7 days of age to insure adequate transmission of virus from their infected mother. The contact, susceptible mother was the nurse in each case. Litters of both infector and contact groups were purposely reduced to 3 to 4 mice in each group. Most litters were weaned at 4 weeks of age, and contacts were separated from infector mice. In several instances, entire litters remained together throughout life.

The results are shown in table 6. The potentialities of the infector mice are revealed in the frequency of and age at death with leukemia. In each of the 34 infector litters, at least one animal had leukemia. The somewhat reduced frequency probably results from an early removal from their high-leukemic mothers, C3Hf*/Lw or C3Hf*/Bi, at from 3 to 7 days of age.

Among the 33 contact litters exposed to transmitted infection, leukemia was found in animals of only 3 litters. Three BALB/c mice, 2 in one litter, showed generalized lymphocytic leukemia at 8½ and 9½ months. Four of 7 of their infector C3Hf*/Gs littermates died with leukemia at 4½ months. One C3Hf/Bi contact mouse died with leukemia at 8½ months. This single animal was caged with 2 C3Hf*/Lw *infector* mice, both of which died with leukemia at 6 and 7 months. Occasionally, mice with early leukemia were found among those in our inbred C3Hf/Bi colony (*12*), but not among BALB/c mice.

To test the possibility that limited infection had occurred within the contact mice, or that a subthreshold virus concentration resulted in inapparent infection, subsequent litters were produced from matings of

Table 6.—Leukemia in susceptible mice kept in contact with viremic, high-leukemic litters

	Infector mice			Contact mice				Foster-nursed mice			
Strain (high leukemic)	Number of litters	Number leukemic / Number of mice	Age at leukemic death (months)	Strain (low leukemic)	Number of litters	Number leukemic / Number of mice	Age at leukemic death (months)	Strain	Number of litters	Number leukemic / Number of mice	Age at leukemic death (months)
C3Hf*/Lw	21	39/62 (63%)	5.6	BALB/c	12	0/41	—	BALB/c	5	12/14 (86%)	6
				C3Hf/Lw	5	0/17	—				
				C3Hf/Bi	2	1/3 [1]	8.5				
						1/61 (1.7%)					
C3Hf*/Bi	13	19/35 (54%)	5.7	BALB/c	11	3/38 [2]	8.7	BALB/c	1	5/5	4.5
				C3Hf/Bi	3	0/13		C3Hf/Bi	3	11/16	7.6
						3/51 (5.9%)				16/21 (76%)	6.6

[1] A litter of 7 mice was born to this C3Hf/Bi female 2 months before signs of leukemia were observed. Four mice became leukemic at an average age of 6 months.

[2] A litter of 3 mice was born to a BALB/c contact female of this group. None became leukemic.

the susceptible BALB/c contacts. Nineteen litters, comprising 79 mice obtained from 10 mothers, remained leukemia-free throughout a 12-month observation period.

Also a sample of BALB/c contact mice was given whole-body X irradiation, 50 r per week for 4 weeks, beginning when the contacts were 1 month of age. This treatment resulted in 1 of 25 contact mice becoming leukemic, whereas 3 of 30 "noncontact" control BALB/c mice similarly irradiated became leukemic.

In contrast to the low frequency of leukemia obtained among susceptible low-leukemic contacts caged with infector mice of similar age, 80% (28 of 35) of the low-leukemic susceptible mice developed leukemia when caged with infectors for the same period but with the high-leukemic C3Hf*/Lw or C3Hf*/Bi mothers of the infector mice. This high frequency of disease resulted from these mice suckling the high-leukemic mothers (*5*) (*see* table 6).

To test whether older infector mice were capable of transmitting MLV infection to young susceptible mice by contact, the mother and her entire litter of low-leukemic mice were caged from birth with 1 to 3 high-leukemic adult males, 1½ to 4½ months old at the beginning of the experiment. Male infector mice were used to preclude transmission of virus to young susceptible mice through the mother's milk. Some of these males had leukemia, and many were in the preleukemic stages of disease during the period the susceptible contact mice were caged with them (*see* table 7). Contacts were separated from the infector males at 5 weeks of age.

Although 68% of the infector males of these high-leukemic lines died with leukemia at an early age, only 1 of 66 susceptible contact mice died leukemic with a generalized lymphocytic neoplasm at 3 months (table 7). This group was observed for 14 months.

The data presented in tables 6 and 7 give evidence of an inability of infector mice to transmit MLV infection regularly or effectively by contact with their susceptible cagemates; yet it was felt that optimal conditions existed for contact infection. The over-all frequency of lymphocytic neoplasms in contact mice was 2.8% (5 leukemics among 178 contacts) over a 14-month observation period. The frequency of disease is similar to that occurring spontaneously in these susceptible mice; however, the age at death from leukemia was considerably shortened.

That the infector mice used in the above two experiments were viremic is shown by two sets of evidence. 1) Virus growth studies[3] of MLV show that the MLV growth curve closely parallels that published for the Rauscher virus (*22*). There is an eclipse phase followed by a progressive increase in virus concentration which begins as early as 7 days (*23*) and gradually reaches a plateau. 2) The results obtained with oral washings and urine inoculations listed in table 8 show that infector mice of the 1- to 6-month-old group were potentially infectious.

---

[3] Personal communication from Dr. J. B. Moloney, National Cancer Institute.

TABLE 7.—Fate of susceptible mice kept in contact from birth with adult viremic male mice

Infector male mice[1]				Contact mice[1]		
Strain (high-leukemic)	Age at contact (months)	Subsequent fate	Strain (low-leukemic)	Number in litter	Number leukemic	Age at leukemic death
C3Hf*/Bi	2	2/3 leukemic at 4–6 months	C3Hf/Bi	8	0	—
C3Hf*/Lw	3	1/2 leukemic at 5 months	C3Hf/Lw	6	0	—
				4	0	—
C3Hf*/Bi	3½	2/3 leukemic at 4–4½ months (1 leukemic during suckling period of contact litter)	C3Hf/Bi	5	0	—
C3Hf*/Bi	3	1/3 leukemic at 8½ months	C3Hf/Bi	6	0	—
C3Hf*/Bi	4½	2/2 leukemic at 5–6½ months (1 leukemic during suckling period of contact litter)	C3Hf/Bi	6	0	—
C3Hf*/Lw	2½	2/3 leukemic at 7–9½ months	C3Hf/Bi	9	0	—
RF*	4	1/1 leukemic at 5 months (leukemic at weaning of litter)	RF			
C3Hf*/Lw	1½	2/3 leukemic at 4–5 months	C3Hf/Lw	3	1	3 months
C3Hf*/Bi	2	2/2 leukemic 3 and 6 months (1 leukemic at weaning of contact litter)	C3Hf/Bi	1	0	—
C3Hf*/Lw	3	1/1 leukemic at 4½ months	C3Hf/Lw	8	0	—
RF*	2½	1/1 leukemic at 4 months	C3Hf/Bi	4	0	—

[1] Number of leukemic infector mice = 17/25 at 5.4 months. Number of contact litters = 12. Number of contact mice = 66.

TABLE 8.—Comparison of infectivity of urine and oral washings from infector donors of different ages

Group	Infector donors	Material inoculated	Recipient mice	Number of litters	Age at inoculation (days)	Number leukemic / Number of mice	Age at leukemic death (range)
A[1]	1) High-leukemic RF* (1 month of age)	Urine (immediate)	BALB/c, RF	3	2–21	5/14 [2]	27% at 6.5 months (3½–9 months)
	2) "	Urine (3 hours)	BALB/c, RF	3	2–6	2/12	
	3) "	Oral washings (immediate)	BALB/c, RF, C3Hf/Bi	5	1–2	11/31	37% at 5.9 months (3½–10½ months)
	4) "	Oral washings (3 hours)	BALB/c, C3Hf/Lw	3	1	8/20	
B	High-leukemic C3Hf*/Lw (4 months of age)	Urine (immediate) 10^{-1} dilution	BALB/c, C3Hf/Bi	10	1–9	16/48	33% at 7 months (4–12 months)
C	High-leukemic RF* and C3Hf*/Bi (preleukemic and leukemic, 4–6 months of age)	Oral washings (immediate)	BALB/c	4	1–3	18/24	75% at 4.4 months (2½–8 months)

[1] In group A, 3 litters of the G8 generation were used; 13 of 17 eventually died with leukemia at 4 to 7 months of age. In group B, 2 litters were used from the G10 generation; 10 of 12 eventually died with leukemia at 4½ to 6½ months of age. In group C, 3 litters of 5 each of the G5 generation were used, but only after leukemia had developed in their littermates. At least one infector mouse in each litter was leukemic at the time of throat swabbing.

[2] Three subsequent litters were obtained from inoculated BALB/c females in group A1; none became leukemic. Leukemic offspring were obtained from 2 of the 5 inoculated mice in group A3. A C3Hf/Bi female inoculated at 2 days of age with oral washings had 9 offspring and 8 were leukemic between the ages of 4 and 5½ months. A BALB/c female inoculated with oral washings at 1 day of age had 5 offspring, 2 of which were leukemic at 6½ and 7 months.

The effectiveness of some leukemogenic viruses introduced orally, either as filtrates in the drinking water (*24*) or through foster nursing, even for a limited time (*6*), suggests that under some conditions transmission of these viruses could occur through contact. Perhaps the unsuccessful attempts to do so in the present study indicate that MLV is extremely labile. However, both urine and oral washings were infective even after 3 hours at room temperature if introduced by the parenteral route (table 8).

The possibility must be considered that exposure to a small quantity of MLV may have provoked an early and actively acquired immunity in the contact mice, although presently no data from studies of MLV support this concept. This is suggested as an explanation for a slight-to-moderate resistance of ICR/Ha Swiss mice, previously caged with infected mice and subsequently challenged with Friend virus (*11*).

MLV is highly concentrated and virulent (*25*). It is more effectively transmitted "vertically" than the other leukemogenic viruses studied and is also apparently less strain-specific. The models employed in the present study should have provided optimal conditions for "horizontal" transmission of this virus. Since little or no evidence of contact infection was obtained, it must be concluded, therefore, that such infections in natural environments must indeed be minimal.

These data confirm the findings of other investigators who used Passage A virus (*26*), Friend virus (*27*), and Graffi virus (*28*). These authors concluded from rather limited studies that there was no evidence of natural infectivity, or, if infectivity occurred, it did so only in rare instances.

REFERENCES

(*1*) Rowe, W. P.: The epidemiology of mouse polyoma virus infection. Bact Rev 25: 18–31, 1961.

(*2*) Rubin, H., Cornelius, A., and Fanshier, L.: The pattern of congenital transmission of an avian leukosis virus. Proc Nat Acad Sci USA 47: 1058–1069, 1961.

(*3*) Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J.: Complement fixation and tissue culture assays for mouse leukemia viruses. Proc Nat Acad Sci USA 53: 931–938, 1965.

(*4*) Law, L. W., and Moloney, J. B.: Studies of congenital transmission of a leukemia virus in mice. Proc Soc Exp Biol Med 108: 715–723, 1961.

(*5*) Law, L. W.: Present status of nonviral factors in the etiology of reticular neoplasms of the mouse. Ann NY Acad Sci 68: 617–635, 1957.

(*6*) Andervont, H. B.: *In utero* transmission of the mouse mammary tumor agent. J Nat Cancer Inst 31: 261–272, 1963.

(*7*) Law, L. W.: Influence of foster-nursing on virus-induced and spontaneous leukemia in mice. Proc Soc Exp Biol Med 111: 615–623, 1962.

(*8*) Law, L. W., Rowe, W. P., Hartley, J. W., and Dawe, C. J.: Further studies on antibody status of mice and response of their litters to parotid-tumor (polyoma) virus. J Nat Cancer Inst 28: 629–647, 1962.

(*9*) Gross, L.: Transmission of mouse leukemia virus through milk of virus-injected C3H female mice. Proc Soc Exp Biol Med 109: 830–836, 1962.

(*10*) Brachet, J.: Formation of the gametes. *In* Chemical Embryology. New York, Interscience Publishers, Inc., 1950, chapt 3, p 87.

(*11*) Mirand, E. A., and Grace, J. T., Jr.: Transmission of Friend virus disease from infected mothers to offspring. Virology 16: 344–347, 1962.

(*12*) Graffi, A.: Neuere untersuchangen über das Virus der myeloischen Leukämie der Maus. Fort Exp Tumorforschung 1: 112–161, 1960.

(*13*) Precerutti, A., and Law, L. W.: Isolation of a murine leukaemogenic virus, P-LLV. Nature (London) 198: 801–803, 1963.

(*14*) Ida, N., Moloney, J. B., and Taylor., G.: Vertical transmission of Moloney leukemia virus in A/LN and BALB/c strains of mice. Gann 56: 239–249, 1965.

(*15*) MacDowell, E. C., and Richter, M. N.: Mouse leukemia. IX. The role of heredity in spontaneous cases. Arch Path (Chicago) 20: 709–724, 1935.

(*16*) Barnes, W. A., and Cole, R. K.: The effect of nursing on the incidence of spontaneous leukemia and tumors in mice. Cancer Res 1: 99–101, 1941.

(*17*) Furth, J., Cole, R. K., and Boon, M. C.: The effect of maternal influence upon spontaneous leukemia of mice. Cancer Res 2: 280–283, 1942.

(*18*) Fekete, E., and Otis, H. K.: Observations on leukemia in AKR mice born from transferred ova and nursed by low leukemic mothers. Cancer Res 14: 445–447, 1954.

(*19*) Fekete, E.: Unpublished data.

(*20*) Kirschbaum, A.: Genetic and certain non-genetic factors with references to leukemia in the F strain of mice. Proc Soc Exp Biol Med 55: 147–149, 1944.

(*21*) Law, L. W.: Maternal influence in experimental leukemia of mice. Ann NY Acad Sci 57: 575–583, 1954.

(*22*) Rauscher, F. J., and Allen, B. V.: Growth curve of a murine leukemia virus in mice. J Nat Cancer Inst 32: 269–275, 1964.

(*23*) Spencer, H. J.: Rapid intracerebral passage and recovery of Moloney leukemia virus from mouse brain tissue. J Nat Cancer Inst 30: 313–321, 1963.

(*24*) Maduros, B. P., Spurrier, W. A., and Schwartz, S. O.: Oral induction of leukaemia in mice. Nature (London) 183: 1816–1817, 1959.

(*25*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*26*) Gross, L.: Studies on pathogenic properties and natural transmission of a mouse leukemia virus. *In* Tumour Viruses of Murine Origin. Ciba Foundation Symposium (Wolstenholme, G. E. W., and O'Connor, M., eds.). London, J. & A. Churchill, Ltd., 1962, pp 159–175.

(*27*) Chamorro, A., Latarjet, R., Vigier, P., and Zajdela, F.: New investigations on the Friend disease. *In* Tumour Viruses of Murine Origin. Ciba Foundation Symposium (Wolstenholme, G. E. W., and O'Connor, M., eds.). London, J. & A. Churchill, Ltd., 1962, pp 176–192.

(*28*) Krischke, W., and Graffi, A.: Untersuchungen zur natürlichen infeketiostät der virusbedingten myeloischen Leukämie der Maus. Acto Biol Med German 5: 413–416, 1960.

DISCUSSION

Dr. Gross: Study of natural transmission of the mouse leukemia virus is of considerable importance and should be followed carefully; it may eventually provide some clue to the epidemiology of this virus. In our experiments thus far performed, we have observed that contact infection among newborn mice exposed to virus-injected littermates resulted in an incidence of leukemia of about 7%, developing later in life in such animals. This incidence is low, but significantly higher than in nonexposed controls. It is of interest that similar results were obtained in experiments performed independently with his virus strain by Graffi, in his laboratory, on the "horizontal" transmission of the mouse leukemia virus. Thus transmission from host to host, particularly among newborns, may occur, but apparently not to any significant degree sufficient to induce a high incidence of disease. The virus is rather transmitted directly through the embryos, from one generation to another, and under certain experimental conditions also through the milk.

Dr. Mirand: We were able to transmit Friend leukemia to offspring from infected mothers (Virology 16: 344–367, 1962) ; however, the incidence was low and the latent period was long. This successful transmission of Friend leukemia is through the mother's milk and has been verified through reciprocal foster-nursing experiments. Transplacental transmission and paternal transmission of Friend leukemia have not been achieved. Urine transmitted Friend virus leukemia with a very low incidence and long latency; oral washings and feces did not. Transmission through cage contact was unsuccessful, also. Therefore, horizontal transmission of Friend virus in the natural environment even under optimal conditions is minimal. Dr. Law, what role does immunity play in the low incidence that one observes in the viral transmission of leukemia?

Dr. Law: I don't know what part it plays. There are some recent reports, as you recall, by the Minnesota group that animals infected with Passage A virus show a disturbed immunologic mechanism in response to T2 bacteriophage, as measured by clearance, and also some disturbance in homograft-rejection mechanisms. But it appears to me that one has to control these experiments to the extent of knowing that you're using a purified virus which you inoculate into the mice. At least some of the preparations we have seen contained 6 or 7 or more recognizable viruses, and I don't see how one can say, therefore, that these results are from Passage A infection.

I am not sure whether Dr. Gross was making a statement or asking a question. I really tried to point out—and maybe I didn't do it very well—that there was a difference in response in the high-leukemic lines we have in the laboratory—AKR, C58, F, C3Hf/Fg, and so on—and the artificially created lines which we have studied. The differences are: One was stable and the other was not; one could show an effect of foster nursing that could not be shown in the established high-leukemic lines; the differences between reciprocal crosses were strikingly different; and there had to be some explanation for the differences we observed in our artificially created lines by MLV and those lines existing for years in the laboratory.

I suggested that perhaps one had to have a mechanism similar to lysogeny because transmission in these established lines is strictly chromosomal, and in the artificially created lines it is strictly extrachromosomal.

Dr. Upton: I should like to ask Dr. Law to help me understand a little more clearly the details of his observations on the conversion of the high-leukemia line to the low-leukemia line. Does this conversion involve an entire litter or does it involve only 1 member of a litter, 1 female, for example?

Also, I thought I understood Dr. Law to say that a mother or a breeding pair that had given rise to low-leukemia offspring might in the subsequent litter retain their high-leukemia tendency. Is this correct?

Dr. Law: Yes. We have observed 9 of these conversions from a high- to a low-leukemic line. In one case these involved members of the entire litter, where either the majority were leukemic or entirely nonleukemic through a period of observation of 12 to 15 months. In one line that we followed which converted spontaneously to a low line, all the members of a prior litter delivered by the mother were leukemic. The subsequent litter converted. The line was carried on from the prior litter as a high-leukemic line. In another case we observed a subsequent litter to the nonleukemic one which became leukemic and was carried on as a high-leukemic line. These are the only two cases we know of where that has occurred.

Dr. Chieco-Bianchi: I have a question for Dr. Law. You did not succeed in inducing vertical transmission of Moloney virus in AKR and C58 mice, is that right?

Dr. Law: Right.

Dr. Chieco-Bianchi: Did you try to induce acceleration of leukemia by injecting newborn mice of these strains with Moloney virus?

Dr. Law: Yes, there is an acceleration of the disease in AKR or C58 after parenteral introduction or foster nursing, but this is not passed on to succeeding generations, *i.e.*, leukemic deaths occurred at 3 and 4 months, for example, in the infected litters, but in the subsequent litters there was a return to the normal pattern of leukemia as far as frequency and latency were concerned.

Dr. Chieco-Bianchi: As to the spontaneous conversion, have you data on the immunologic state of such resistant mice? In other words, can an immune reaction be detected, both humoral or cellular, against the virus?

Dr. Law: The role of immunologic competence is difficult to decide because the organ affecting immunologic competence in mammals is also the organ that becomes involved in the leukemogenic process.

Dr. Furth: I really see no basic difference between the concepts of Dr. Law and Dr. Gross. Dr. Law clarified in his comment where the apparent difference lies, stating that the AKR situation is analogous to lysogeny. To my knowledge, no one has ever converted the AKR strain to a nonleukemic one. Would he please clarify this further. How do you conceive this lysogenic modification? Would virus be incorporated in the lymphoid cell which is then perpetuated? Dr. Latarjet speculated on lysogeny and viral leukemogenesis, and we hope to hear him discuss them further.

One of the most interesting concepts you introduced in your lecture relates to this contrasting of the strains in which the virus is ever present—as in AKR mice—and others in which virus is introduced and in which immunologic phenomena, apparently different from those occurring in AKR mice, play a prominent role in pathogenesis.

Dr. Latarjet: In fact, we spent a lot of time in our Institute to see whether there is a lysogenic situation in the AKR mouse. These experiments have been published and discussed in detail. The end result was that we have no evidence whatsoever that there is a lysogenic situation; conversely, we have some good experimental evidence that the AKR embryo contains all the antigens of the virus; this means that the embryo is infected with complete virions and not with a provirus—the nucleic acid of the virus, only.

That is why I am very much interested in the suggestion Dr. Law formulated that after infection the vertical transmission should be accounted for by a lysogenic situation which would contrast with that present in the permanent strains with high spontaneous incidence. This point could be checked, as we did, by some tolerance experiments using embryo extract to see if they can induce tolerance to the virus—to superinfection by the virus in another strain.

Dr. Law: I suggested that some mechanism similar to lysogeny must occur in the high-leukemic strains, since it is quite clear that transmission must be chromosomal and it must be by the spermatozoa. If you cross C3H animals by AKR males, the transmission must occur through the spermatozoa, since frequencies in reciprocal crosses are similar.

Several Aspects of Vertical Transmission of Moloney Virus[1]

Noriaki Ida, *M.D.*, Arimitsu Fukuhara, *M.D.*, *and* Yasuhiro Ohba, *M.D.*, *Toyo Kogyo Hospital, Hiroshima, Japan*

THE experiments reported by Ida, Moloney, and Taylor (*1–3*) were undertaken to investigate the possible natural modes of transmission of Moloney leukemia virus (MLV) in A/LN and BALB/c strains of mice.

EMBRYONAL TRANSMISSION OF VIRUS IN A/LN MICE

The leukemia virus was injected intravenously in a 0.2 ml dose into pregnant A/LN mice at 8 stages of gestation, *i.e.*, 1, 4, 7, 9, 11, 13, 15, and 18 days (table 1). The day of gestation was estimated from the time of first detection of the vaginal plug. Prior to delivery, wire mesh was placed in the cages. At birth, the offspring were immediately given to normal BALB/c foster nurses to avoid the possible transmission of either virus or its antibody through the milk of the inoculated mothers.

Although 88 females received the leukemia virus at various stages of pregnancy, only 23 carried pregnancy to term; the others aborted after injection of the virus. Leukemia developed in 11 offspring of 23 mothers inoculated either before or after the placenta had formed.

The incidence of leukemia within each litter ranged from 16.7 to 100%, and the latent periods ranged from 31.6 to 59.4 weeks. Nine of the 23 mothers inoculated with the virus during pregnancy did not develop the disease during the period of observation, although their offspring (5 animals) died with the induced disease. This observation may be significantly related to similar clinical experiences.

EMBRYONAL TRANSMISSION OF LEUKEMIA VIRUS IN BALB/c MICE

A similar experiment was conducted in BALB/c mice. No living offspring were obtained from mothers who had received virus injections during early stages of gestation, *i.e.*, days 1, 4, and 7.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

Table 1.—Vertical transmission of Moloney leukemia virus in A/LN mice

	Mother				Leukemia in offspring						
					Tabulation on basis of mother group			Tabulation on basis of gestation group			
Period of inoculation	Day of gestation	Number of mothers	Leukemia in mother	Individual latent period (weeks)	Average latent period (weeks)	Number of leukemic animals / Number of animals alive at earliest appearance of leukemia	Incidence of leukemia (%)	Average latent period (weeks)	Number of leukemic animals / Number of animals observed	Incidence of leukemia (%)
Preplacental	1	15	—	NL*	59. 4	1/2	50. 0	59. 4	2/15	13. 3
	1	33	+	59. 9	—	0/2	0			
	1	3	+	45. 9	59. 4	1/6	16. 7			
	1	38	—	NL	—	0/5	0			
	4	19	—	NL	—	0/3	0	52. 2	1/4	25. 0
	4	17	+	39. 3	52. 2	1/1	100. 0			
Postplacental	9	83	+	59. 0	58. 3	1/3	33. 3	57. 4	2/7	28. 6
	9	73	+	53. 9	56. 6	1/4	25. 0			
	11	74	+	46. 4	58. 4	1/5	20. 0	43. 8	4/26	15. 4
	11	13	—	NL	31. 6	1/4	25. 0			
	11	36	+	27. 4	—	0/3	0			
	11	84	+	27. 6	—	0/3	0			
	11	82	—	NL	—	0/3	0			
	11	76	+	47. 0	—	0/3	0			
	11	72	—	NL	41. 5	2/5	40. 0			
	13	58	+	54. 1	—	0/5	0	37. 6	2/22	9. 1
	13	46	+	54. 1	—	0/6	0			
	13	39	+	39. 6	41. 0	1/5	20. 0			
	13	56	—	NL	34. 1	1/4	25. 0			
	13	24	—	NL	—	0/2	0			
	15	64	+	24. 0	—	0/2	0	—	0/7	0
	15	60	—	NL	—	0/5	0			
	18	35	+	54. 4	—	0/2	0	—	0/2	0

*NL: Nonleukemic at autopsy.

Eight of the BALB/c animals, among the 68 mice born of females inoculated at gestation days 9, 11, 13, 16, 17, 18, 19, and 20, which were alive at the first manifestation of leukemia, developed the disease. The incidence within each litter ranged from 10 to 100%. The average latent period ranged from 19.6 to 67.4 weeks (table 2).

Thus these phases of the study indicated that this agent can be transmitted to the embryos of BALB/c and A/LN mice through the placental barrier.

EMBRYONAL TRANSMISSION OF LEUKEMIA VIRUS AT 12TH DAY OF GESTATION IN BALB/c MICE

Twelve BALB/c females received the virus on the 12th day of gestation of the second pregnancy. Of 102 offspring, 65 survived. These offspring were foster-nursed by normal BALB/c mice. Five of the 51 in this group (9.8%), alive at the time of the first appearance of leukemia, developed leukemia. The incidence within each litter ranged from 25 to 100%. The latent period ranged from 36.4 to 55.3 weeks (table 3).

No leukemia developed in the 201 control animals (offspring from the first litters) during an observation period of more than 1 year.

EFFECT OF RECIPROCAL FOSTER-NURSING: TRANSMISSION OF VIRUS THROUGH MOTHER'S MILK IN BALB/c MICE

To determine additional routes of transmission of the virus from mother to offspring, reciprocal foster-nursings were carried out. Mothers receiving the virus at the 12th gestation day foster-nursed a number of mice obtained from normal BALB/c mothers. Of the 72 offspring surviving until the first observation of leukemia, 15 (20.8%) developed the disease at an average age of 39.5 weeks. All except 2 of the foster mothers given the virus developed leukemia (table 4).

These results confirm and extend previous findings (*4*, *5*) that the leukemia virus can be transmitted to successive generations through the milk of viremic mothers.

Thus evidence was obtained for the transfer of a murine leukemia virus across extraembryonic membranes. The data indicate that the virus will infect the embryos prior to the formation of the placenta. Similarly, the virus will cross the placental barrier and infect the fetus. It is interesting that several of the mothers remained free from the disease through the period of observation, whereas their offspring died with the virus-induced neoplasm.

Table 2.—Vertical transmission of Moloney leukemia virus in BALB/c mice

Mother				Leukemia in offspring							
				Tabulation on basis of mother group				Tabulation on basis of gestation group			
Day of gestation	Number of mothers	Leukemia in mother	Individual latent period (weeks)	Average latent period (weeks)	Number of leukemic animals /	Number of animals alive at earliest appearance of leukemia	Incidence of leukemia (%)	Average latent period (weeks)	Number of leukemic animals /	Number of animals observed	Incidence of leukemia (%)
9	59	+	30. 4	—	0/2		0	67. 4	1/5		20. 0
9	61	+	19. 3	—	0/2		0				
9	56	+	30. 3	67. 4	1/1		100. 0				
11	38	+	17. 9	66. 4	1/7		14. 3	66. 4	1/13		7. 7
11	49	+	38. 7	—	0/3		0				
11	52	+	28. 1	—	0/1		0				
11	46	+	23. 1	—	0/2		0				
13	66	−	NS*	—	0/3		0	42. 3	2/5		40. 0
13	69	+	37. 1	42. 3	2/2		100. 0				
16	78	+	25. 7	26. 4	1/2		50. 0	23. 0	2/9		22. 2
16	9	+	26. 0	—	0/1		0				
16	83	+	24. 0	—	0/1		0				
16	7	+	20. 3	19. 6	1/5		20. 0				
17	79	+	20. 7	—	0/4		0	—	0/10		0
17	80	+	20. 0	—	0/3		0				
17	6	−	Killed	—	0/3		0				
18	5	+	17. 9	—	0/3		0	—	0/6		0
18	76	+	15. 9	—	0/3		0				
19	20	−	NS	—	0/6		0	37. 7	1/10		10. 0
19	1	−	—	—	0/2		0				
19	4	−	NS	37. 7	1/2		50. 0				
20	19	+	16. 9	27. 4	1/10		10. 0	27. 4	1/10		10. 0

*NS: Nonspecific. Animals died from causes other than leukemia, as shown in the following example.

TABLE 3.—Vertical transmission of leukemia virus at the 12th day of gestation in BALB/c mice foster-nursed by normal BALB/c mother (reciprocal foster-nursing experiment)

Mother				Leukemia in offspring					
				Tabulation on basis of mother group			Tabulation on basis of gestation group		
Day of gestation	Number of mothers	Leukemia in mother	Individual latent period (weeks)	Average latent period (weeks)	Number of leukemic animals / Number of animals alive at earliest appearance of leukemia	Incidence of leukemia (%)	Average latent period (weeks)	Number of leukemic animals / Number of animals observed	Incidence of leukemia (%)
12	8	+	27. 6	—	0/3	0	51. 2	5/51	9. 8
	22	−	NS*	—	0/6	0			
	33	+	11. 9	55. 3	1/3	33. 3			
	38	−	NS	—	0/7	0			
	32	+	16. 1	55. 3	1/4	25. 0			
	40	−	—	—	0/4	0			
	26	+	33. 1	55. 0	1/1	100. 0			
	44	+	34. 9	—	0/5	0			
	9	+	11. 4	—	0/3	0			
	23	+	27. 9	54. 0	1/4	25. 0			
	75	+	22. 9	36. 4	1/3	33. 3			
	67	−	NS	—	0/8	0			

* NS: Nonspecific. Animals died from causes other than leukemia.

TABLE 4.—Transmission of leukemia virus through the mother's milk in BALB/c mice (reciprocal foster-nursing experiment)

Mother						Leukemia in offspring					
Parent mother			Foster mother inoculated			Tabulation on basis of mother group			Tabulation on basis of whole litter		
Number of mothers	Leukemia in mother	Individual latent period (weeks)	Number of mothers	Leukemia in foster mother	Individual latent period (weeks)	Average latent period (weeks)	Number of leukemic animals / Number of animals observed	Incidence of leukemia (%)	Average latent period (weeks)	Number of leukemic animals / Number of animals observed	Incidence of leukemia (%)
45	—	NL*	8	+	27. 6	—	0/5	0	39. 5	15/72	20. 8
68	—	NL	22	—	NS†	37. 2	3/8	37. 5			
72	—	NL	30	+	35. 1	21. 1	1/7	14. 3			
73	—	NL	33	+	11. 9	30. 6	2/8	25. 0			
77	—	NL	38	—	NS	55. 3	1/8	12. 5			
10	—	—	32	+	16. 1	—	0/6	0			
76	—	NL	26	+	33. 1	—	0/5	0			
62	—	NL	44	+	34. 9	—	0/2	0			
5	—	NL	9	+	11. 4	—	0/3	0			
41	—	NL	23	+	27. 9	54. 9	3/5	60. 0			
58	—	—	24	+	25. 4	33. 1	3/3	100. 0			
18	—	NL	75	+	22. 9	—	0/4	0			
60	—	NL	1	+	24. 9	44. 5	2/4	50. 0			
71	—	NL	20	+	34. 4	—	0/4	0			

*NL: Nonleukemic at autopsy.

†NS: Nonspecific: Animals died from causes other than leukemia.

NATURAL VERTICAL TRANSMISSION OF MOLONEY LEUKEMIA VIRUS IN SWISS MICE

Following the experiment on vertical transmission of MLV reported previously (*1–3*), we studied a natural transmission of MLV in Swiss mice. The incidence of spontaneous leukemia in Swiss mice in our mouse colony was less than 1.0% during 2 years' observation.

Moloney virus was inoculated into newborn Swiss mice. Brothers and sisters were mated when they reached adult age. Their offspring were fostered by their own mothers. Thirty (21.7%) of 138 Swiss mice born to virus-infected parents developed leukemia, with a latent period ranging from 50 to 245 days. The disease developed in 35% (21/60) at the first, 16% (9/57) at the second, and 0% (0/21) at the third generation. The incidence of leukemia in subsequent generations seemed to reduce gradually during this natural vertical transmission of the leukemia virus in this particular strain of mice.

If mice from the virus-infected mothers were fostered by normal intact mothers of the same strain, none developed the disease. No leukemias were found in 24 mice in the first generation, 41 in the second, and 8 in the third, except for 1 animal that developed leukemia at 243 days of age in the first generation.

Although the Swiss mice seemed less susceptible to the virus in the mother's milk, we observed one strain that showed leukemia in 5 successive generations: 2 (100%) of 2 offspring in the first, 5 (56%) of 9 in the second, 5 (83%) of 6 in the third, 1 (20%) of 5 in the fourth, and 1 (25%) of 4 in the fifth generation. The latent period ranged from 80 to 157 days. However, this strain was exceptional.

Interestingly, both the first and second litter from a virus-infected mother developed leukemia though the mother was not leukemic when the first litter was nursed. When the second litter was nursed, the mother was obviously leukemic. There were no remarkable differences in the incidence of leukemia between the first litter (5/10, at 85–248 days of age) and the second litter (2/7, at 59–86 days of age). These experiments show obviously that leukemia was transmitted through the milk of the isologous mother.

Leukemia was also transmitted through the milk of a homologous mother. Normal Swiss mice fostered by a virus-infected BALB/c mother developed leukemia (3/5 animals, at 93–165 days of age). The agent was transmitted to the second generation in Swiss mice (3/4, 90–120 days of age) in this experiment. In another similar experiment, the disease was induced by homologous mother's milk (4/4, 87–124 days of age) in the same way. It was our experience that 10 days' foster-nursing was sometimes long enough to induce the disease in offspring.

There were no leukemias in 19 offspring obtained from normal females mated with virus-infected males. Paternal virus transmission seems ineffective.

In addition to these experiments described, the possibility of horizontal transmission of the virus through excreta was studied. Extracts of (*a*) mucous membranes of small intestine, (*b*) contents of intestine of leukemic mice, and (*c*) pooled stools and urine from several leukemic mice were inoculated into newborn Swiss mice to see if leukemia would develop in the recipients. Two of 100 animals in group (*a*) and 1 of 33 in group (*b*) developed leukemia at 9 months of age, but none of 30 animals in group (*c*) developed the disease. Negative results were also obtained from 24 control animals inoculated with heated extracts (at 56° C, for 30 min) of the stool and urine during 10 months' observation. The results of this experiment seem insignificant.

The virus particles were demonstrated electron microscopically (*6*) in the various tissues of the animals with leukemia induced by the mother's milk: 1) cylindrical or tubular particles in splenic tissues, 2) virus particles in inclusion-like bodies in the infected mammary gland, 3) "tail" shape of the virus particle in ovarian tissue and mammary gland, and 4) mature viral particles in the mammary gland and milk of the infected mothers.

Thus we (*7*, *8*) confirmed natural transmission of MLV via mother's milk (*4*, *5*, *9–12*) in mice, biologically and electron microscopically.

CHROMOSOME COMPLEMENTS IN THE SWISS MOUSE DURING INFECTION WITH MOLONEY LEUKEMIA VIRUS

Studying the transmission of this virus-induced leukemia, we found increasing evidence that, contrary to previous studies, there might be chromosomal alterations in virus-induced tumors. Therefore, we began a cytogenetic study of the tissues of mice with MLV-induced leukemia. The chromosomal examination of the cells of various organs in these leukemic mice "naturally" infected by the virus yielded information about the distribution of the chromosome number, including variations in ploidy, as well as data on the structural alterations in the chromosomes of these animals. We further investigated the possibility of an extra-embryonic transmission of the agent in murine animals; the details of these experiments will be discussed later.

Chromosome studies on virus-induced leukemia in mice have been conducted by many investigators: spontaneous leukemia in AK (*13*) and AKR mice (*14–17*), induced leukemia with S37 and Friend virus (*18*), with Graffi, Gross, and Friend viruses (*19*), Friend and Rauscher viruses (*20*), Moloney virus (*21*), and Rich virus (*22*).

Our experiments were initiated because chromosomal alterations could be demonstrated in the process of the vertical transmission of leukemia virus.

Virus stocks were prepared from material generously supplied by Dr. John B. Moloney, National Cancer Institute, Bethesda, Maryland.

For chromosome studies, mice were fostered by the virus-infected mothers or inoculated intraperitoneally with 0.1 ml of undiluted MLV at birth. Many of the mice infected showed readily palpable, enlarged spleens in 3 or 4 months. One-day-old animals were also tested for chromosomes for study of a possible placental transmission of leukemia virus.

The Swiss mice used originated from the Rockefeller Institute, New York City, and were raised at Hokkaido University in Japan since 1953. The C3H mice were supplied in 1962 by the courtesy of Dr. Moloney.

Mice were given 0.3 ml of 0.025% colchicine intraperitoneally 1 or 2 hours before they were killed by cervical dislocation. Chromosomes were prepared by a modification of the method described by Fox and Zeiss (*23*).

CHROMOSOMES IN NEWBORN MICE OBTAINED FROM THE MOTHER INOCULATED WITH VIRUS DURING PREGNANCY

Evidence was obtained in our previous work (*1*) that leukemias developed in offspring whose mothers were inoculated with the virus during pregnancy. In addition, Ingalls *et al.* (*24*) recently reported that chromosomal anomalies were induced in mice by injection of a certain teratogen (6-aminonicotinamide) in pregnancy. Since we wanted to know if possible chromosome changes did occur in fetuses that might be infected with the virus during pregnancy, the following experiment was conducted: Several litters of Swiss and C3H mice were obtained from mothers inoculated with the virus during pregnancy. The liver and thymus of 9 newborn Swiss and 5 newborn C3H mice were tested for chromosome changes soon after birth. At the same time, 2 litters (Swiss) of 4 animals and 1 litter (C3H) of 2 animals were also tested as controls.

As shown in table 5 and figures 1 and 2, the cells in the liver, thymus, and spleen of these animals showed a normal diploid complement of 40, and no chromosomal aberrations were observed in either the Swiss or the C3H mice.

Table 5.—Number of chromosomes in offspring from the mother given the virus during pregnancy

	Mouse No. and strain	Gestation period at virus injection	Age of mouse when killed (days)	Organs	Number of cells with indicated chromosome number								Total No. of cells
					39	40	41	42	43	44	80	160	
Control	1(SW)		1	Liver		54							54
	2(SW)		1	Liver		51						1	52
				Thymus	1	48					1		50
	3(SW)		1	Liver	1	55					2		58
	4(SW)		1	Liver		50							50
				Thymus		25							25
	5(C3H)		1	Liver	1	48					2		51
	6(C3H)		1	Liver	1	48							49
Experiment	1(SW)	12	1	Liver		37							37
				Thymus	2	16							18
	2(SW)	12	1	Liver		37							37
				Thymus	3	17							20
	3(SW)	12	1	Liver	5	38					1		44
				Thymus	4	33							37
	4(SW)	13	1	Liver	1	36							37
				Thymus	2	32							34
	5(SW)	15	1	Liver		32							32
				Thymus		32						3	35
	6(SW)	15	1	Liver	2	26							28
	7(SW)	15	1	Liver	1	37							38
	8(SW)	15	1	Thymus	2	25							27
	9(SW)	18	3	Liver	11	46					5		62
				Spleen	6	27							33
	10(C3H)	13	1	Liver		50							50
	11(C3H)	13	1	Liver	1	48							50
				Spleen	1	50							51
	12(C3H)	13	1	Liver		50							50
	13(C3H)	13	1	Liver	1	49							50
	14(C3H)	13	1	Liver		50							50

CHROMOSOME IN EARLY DISEASE

Various organs such as bone marrow and pooled spleen of 5 mice—20 days after inoculation at birth—and bone marrow, spleen, thymus (right and left, separately), and lymph nodes of 4 mice—50 days after inoculation of virus at birth—were examined for chromosome changes. As shown in table 6, most of the organs in these 9 animals tested showed a normal diploid complement, and no other chromosomal alterations were found.

Table 6.—Chromosome number of virus-inoculated mice, 20 and 50 days after inoculation at birth

Mouse No.	Sex	Age of mouse when killed (days)	Organs	Number of cells with indicated chromosome number							Total No. of cells
				39	40	41	42	43	44	80	
1	M	20	Bone marrow	6	59					1	66
2	M	20	Bone marrow	2	34	1					37
1–2			Pooled spleen	2	28						30
3	F	20	Bone marrow	3	30					1	34
4	F	20	Bone marrow	4	34						38
5	M	20	Bone marrow	6	30						36
3–5			Pooled spleen	5	25						30
6	F	50	Bone marrow		36					1	37
			Spleen		37						37
			Thymus, right		37						37
			Thymus, left	2	35						37
7	F	51	Bone marrow		37						37
			Spleen	2	42						44
			Thymus, right		50						50
			Thymus, left		37						37
			Cervical lymph nodes		36						36
			Mesenteric lymph nodes		37						37
8	F	51	Bone marrow		37						37
			Spleen		37						37
			Thymus, right	1	36						37
			Thymus, left	1	36						37
			Mesenteric lymph nodes	1	19						20
9	M	53	Bone marrow		37						37
			Spleen		37						37
			Thymus, right	1	36						37
			Thymus, left	2	35						37

CHROMOSOMES OF NONLEUKEMIC OFFSPRING OBTAINED FROM VIRUS-INFECTED PARENTS, NURSED EITHER BY VIRUS-INFECTED OR NORMAL MOTHER

As shown in tables 7 and 8, no chromosome changes in bone marrow cells were observed in 7 mice obtained from virus-infected parents and nursed by the infected mother; the other 9 mice were from the infected parents and nursed by normal mice. In these nonleukemic animals, chromosome changes could not be detected.

TABLE 7.—Chromosome study of nonleukemic offspring obtained from virus-infected parents and nursed by normal mother

Mouse No.	Sex	Age of mouse when killed (days)	Organs	Number of cells with indicated chromosome number								Total No. of cells
				39	40	41	42	43	44	80–82	160	
1	M	601	Bone marrow		35						1	36
2	F	601	Bone marrow		43							43
3	F	527	Bone marrow		43							43
4	M	527	Bone marrow	2	36							38
5	M	527	Bone marrow		40							40
6	F	641	Bone marrow		31							31
			Spleen		12							12
7	F	641	Bone marrow	1	47							48

TABLE 8.—Chromosome study of nonleukemic offspring obtained from virus-infected parents and nursed by virus-infected mother

Mouse No.	Sex	Age of mouse when killed (days)	Organs	Number of cells with indicated chromosome number							Total No. of cells
				39	40	41	42	43	44	160	
1	M	606	Bone marrow	4	36						40
2	M	608	Bone marrow	1	35						36
3	M	608	Bone marrow		39						39
4	M	543	Bone marrow	1	34						35
5	F	543	Bone marrow	4	31						35
6	F	543	Bone marrow	1	34	1				1	37
7	F	543	Bone marrow		40						40
8	F	419	Bone marrow		70						70
9	F	418	Bone marrow	1	69						70

CHROMOSOME CHANGES LATE IN DISEASE

Mice fostered by the virus-infected females or inoculated with the virus at birth developed markedly enlarged thymus, lymph nodes, and spleen. These animals surviving the initial viremia developed leukemia later. Analyses were performed on animals late in the course of the disease to follow the chromosome changes.

With Moloney leukemia, the chromosome number of bone marrow, spleen, lymph nodes, thymus, and liver cells (in some animals) was determined after a latent period ranging from 61 to 129 days, except for several animals in which the latent period was more than 6 months. The chromosome number observed in spleen cells of 15 leukemic mice is summarized in table 9.

Eight of 15 spleens had cells with a predominantly normal diploid chromosome complement, while 7 of 15 spleens had cells predominantly hyperdiploid. Two types of mode in the chromosome number (40 and 44, or 40 and 41) were observed in 6 of the 15 spleens examined. The spleen of

TABLE 9.—Number of chromosomes in spleen cells of mice with leukemia induced by Moloney virus

	Mouse No.	Sex	Age of mouse when killed (days)	Number of cells with indicated chromosome number								Total No. of cells
				39	40	41	42	43	44	58	80–82	
Control	1	F	60–90		50							50
	2	F	"		36							36
	3	F	"		40							40
	4	M	"		37							37
	5	M	"		37							37
	6	M	"		35							35
Experiment	1	M	110	2	13	1	5	6	17	1	2	47
	2	F	81	8	36	38	7				2	91
	3*	M	124	5	32							37
	4	M	87		20	10						30
	5	M	74	9	14	41	4				2	70
	6	F	96	1	33							34
	7	M	61		22	2						24
	8	M	97		40							40
	9*	M	95	3	67	12	12	3			5	102
	10*	F	119	2	36							38
	11	F	88	2	31							33
	12	F	129		31							31
	13*	M	458	2	24	12	1					39
	14*	F	274	4	28	7	1					40
	15	M	194		29	4						33

*Indicates mice fostered by virus-infected mother.

#1 male mouse consisted of predominantly 40 and 44; the spleens of #2 female, #4 male, and #13 male consisted largely of cells with 40 and 41 chromosomes. The same tissue of #5 male contained predominantly cells with 41 chromosomes, and #9 male contained 40, 41, and 42 chromosomes. Polyploid cells were observed in #1 male, #2 female, #5 male, and #9 male, for which the chromosome number is 80 to 82.

The chromosome count on spleen cells of six 2- to 3-month-old normal Swiss mice was included as a control. These numerical chromosome alterations were observed in about half of the virus-induced leukemias (7/15) in Swiss mice.

Some variations in ploidy in different tissues were observed. An interesting finding is that, in 2 mice (#9 male and #14 female) with leukemia induced by the mother's milk, the enlarged thymus showed a diverse distribution of chromosome number, and polyploid cells were more frequently observed in the thymus than in the spleen and bone marrow (table 10).

Karyotypic changes such as metacentrics, small, and long chromosomes were observed in these leukemic animals. Figure 3 shows 81 chromosomes in a spleen cell examined in #9 male which was fostered by the virus-infected mother and developed leukemia 95 days after birth. Two diploid cells in spleen tissue had a long telocentric chromosome (fig. 4) in #14 female. However, these chromosome changes were detected only in the late stages of leukemia.

TABLE 10.—Number of chromosomes in various infiltrated organs of leukemic mice

Mouse No.	Sex	Organs	Number of cells with indicated chromosome number										Total No. of cells
			39	40	41	42	43	44	58	76	80–82	162	
1	M	Spleen	2	13	1	5	6	17	1		2		47
		Bone marrow	1	16	4	7	9	33					70
2	F	Spleen	8	36	38	7					2		91
		Bone marrow	2	23	2	1							28
3*	M	Spleen	5	32									37
		Thymus		35	1								36
		Lymph nodes	2	30	1								33
5	M	Spleen	9	14	41	4					2		70
		Bone marrow	5	18	38	5					4		70
9*	M	Spleen	3	67	12	12	3				5		102
		Thymus	1	72	3	1					23		100
		Bone marrow	3	63	3	7							76
10*	F	Spleen	2	36									38
		Thymus	13	19	1	1							34
		Lymph nodes	4	29	1								34
		Liver	4	27	1					1		1	34
		Bone marrow	3	28									31
12	F	Spleen		31									31
		Thymus	2	30									32
		Bone marrow	1	29									30
13*	M	Spleen	2	24	12	1							39
		Lymph nodes		31	33	4							68
		Bone marrow	3	36									39
14*	F	Spleen	4	28	7	1							40
		Thymus	3	23	20	7	2				14		69
		Bone marrow	3	53							1		57
15	M	Spleen		29	4								33
		Lymph nodes		20	4								24
		Bone marrow		34	1								35

*Indicates mice fostered by virus-infected mother.

CHROMOSOMES IN NEWBORNS FROM MOTHERS INOCULATED DURING PREGNANCY WITH LEUKEMIA CELLS WITH 41 CHROMOSOMES

One C3H mouse (#58 female) showed predominantly 41 chromosomes in various organs such as liver, mesenteric lymph nodes, spleen, and ascites at the third transplantation of leukemic cells originating from leukemia induced by the mother's milk (table 11 and fig. 5).

To investigate a possible transmission or metastasis of leukemic cells into the fetuses from the mother, the cells with 41 chromosomes were injected intravenously in a dose of 1.0 ml ($3.3–8.1 \times 10^4/\text{mm}^3$) into 5 pregnant C3H mice at the 13th, 15th, 16th, 17th, and 18th day of gestation. Twenty newborns from 5 mothers thus treated were examined for chromosome number. The liver tissues in most of the mice and the thymus in some were examined.

Nineteen newborns showed a normal diploid cell in the organs tested. However, 1 of 6 in a certain litter showed 41 chromosomes in 167 of the 170 liver cells examined (table 12 and fig. 6). In contrast to this, such a

TABLE 11.—Number of chromosomes in transplanted leukemia (MLV) of C3H #58 female

Sex	Age of mouse when killed (days)	Organs	Number of cells with indicated chromosome number								Total No. of cells
			39	40	41	42	43	44	80–82	160	
F	97	Liver		2	48	1					51
		Mesenteric lymph nodes		1	49						50
		Bone marrow		37	15				1	3	56
		Spleen		1	48	1	1				51
		Ascites			36				14		50

high percentage of hyperdiploid cells was not observed in the liver tissue of normal newborns. However, before any conclusions on the metastasis of leukemic cells from mothers to their offspring can be made, further study must be done.

DISCUSSION

Gross (*25*) observed that filtrates of embryos from AK mice, as well as filtrates of ovaries and testes of AK mice (*26*), induced leukemia when inoculated into C3H mice at birth. From these results this investigator reasoned that leukemia in mice was an "egg-borne virus disease" and that the effective agent could be transmitted from one generation to the next through the embryos. Thus the possibility of "vertical transmission" of a leukemia virus was suggested.

Dmochowski *et al.* (*27*) recently demonstrated the presence of mature leukemia virus particles in the spleen and thymus of these animals. Previously, the Passage A virus, a more active line of the Gross agent, was shown to be transferred through the maternal line of mice, more particularly through the milk of infected mothers (*10–12*).

The ova transfer method was used by Fekete and Otis (*28*) to investigate the possibility of the uterine environment affecting the natural transmission of a leukemia agent. They concluded that extrinsic factors, such as the influence of nursing by foster mothers of a low leukemia strain and the combined influence of such nursing and uterine environment in low leukemia strain mothers, do not alter the incidence of leukemia and the expression of genetic tendencies.

Thus in the interpretation of the studies described herein, the following should be considered: 1) Are the eggs directly infected by the injected virus? 2) Are the embryos contaminated by the virus by circulation of the mother's blood? 3) Are the embryos attacked by the virus after placental formation?

In the present experiments, leukemia developed in the offspring of A/LN strain mothers given injections of virus during an early stage of gestation

TABLE 12.—Number of chromosomes in newborns from mother* inoculated with leukemia cells with 41 chromosomes

Experiment	Mouse No.	Gestation period at leukemic cell injection	Age of mouse when killed (days)	Organs	Number of cells with indicated chromosome number									Total No. of cells
					39	40	41	42	43	44	80–82	160	163	
I	1	16	1	Liver		1	167				1		1	170
	2	(3.3×10^4)†	1	Liver	5	43						2		50
	3		1	Liver	1	49								50
	4		2	Liver	3	30								33
	5		2	Liver	1	49								50
	6		2	Liver	1	49								50
	4–6		2	Pooled thymus		10								10
II	7	18	1	Liver		48					2			50
	8	(5.8×10^4)	1	Liver	1	48					1			50
	7–8		1	Pooled thymus	1	48					1			50
III	9	18	1	Liver	1	34								35
	10	(5.3×10^4)	1	Liver		35								35
				Thymus		35								35
	11		1	Liver	2	35								37
				Thymus		35								35
IV	12	13	1	Liver		55								55
	13	(8.1×10^4)	1	Liver		51								51
	14		1	Liver		42								42
V	15	17	1	Liver	1	36								37
	16	(5.4×10^4)	1	Liver		35					2			37
	17		1	Liver		37								37
	18		1	Liver		37								37
	19		1	Liver	1	35								36
	20		1	Liver		37								37
	15–20		1	Pooled spleen	3	33					1			37
			1	Pooled thymus	4	33								37

* Note: Mother of offspring I_{1-6} had 50 spleen and 50 bone marrow cells examined; 49 cells from each tissue had 40 chromosomes; 1 cell from each tissue had 39.

† Numbers in parentheses indicate the number of cells per mm ^{3}.

as well as in late pregnancy. According to Law (*4, 29*) although effective transmission of virus across placental membranes occurs rarely, of the 51 C3H mice from 14 litters born to MLV-infected mothers (G_0–G_2) but foster-nursed by normal mothers, 7 offspring developed leukemia at latent periods ranging from 5 to 13 months. Two were granulocytic leukemia and one was a reticulum cell sarcoma, type A. Thus a natural transmission of virus during the prenatal period was observed in C3H strains, although its incidence was low and sporadic in his experiment.

In our study, not all the mothers receiving the virus developed leukemia, although the disease occurred in some of their offspring. Similar results were also obtained in Law's experiment (*4*): Virus-infected C3H females, either those inoculated during the neonatal period (G_0) or receiving MLV from their mothers (G_1–G_5), transmitted virus to their fostered litters prior to development of leukemia. Two mothers that died nonleukemic at 12 and 13 months transmitted MLV to their fostered litters.

In the reciprocal foster-nursing experiments, the incidence of leukemia in BALB/c mice was lower than that described by Law and Moloney (*9*), and Law (*4*). In this respect, possibly BALB/c mice are less susceptible to the leukemic agent in the milk than are C3H sublines, or propagation of the virus in the mammary gland was poor in the BALB/c mice. However, the data obtained strongly indicate that transmission of the Moloney virus through the mother's milk appeared to be the most effective route of natural transfer.

In our later experiment of the natural vertical transmission of MLV in Swiss mice, virus infectivity seems to be reduced during viral transmission through generations, *i.e.*, leukemia developed in 35% (21/61), 16% (9/57), and 0% (0/21) at the first, second, and third generation, respectively. Since the foster mothers after the second generation were infected with the virus, possibly exposure to a small quantity of MLV may have provoked either actively acquired immunity in these animals or passively acquired immunity from the virus-infected mothers through the mother's milk. Similar results were also found with the Friend virus (*30*) and by Krischke and Graffi (*31*) with the virus of myeloid leukemia.

During our studies on the transmission of this agent, chromosomal changes were noted in the cells of the bone marrow, spleens, and other organs of leukemic mice "naturally" infected by the virus. These studies have been extended to the investigation of a possible extraembryonic transmission of the agent in mice. In our previous work (*1*), it was evident that leukemia developed in offspring whose mothers were inoculated with the virus during pregnancy. Since we wanted to know if possible chromosome changes occurred in fetuses that might be infected with the virus during pregnancy, newborn mice obtained from the mother inoculated with the virus during pregnancy were examined for chromosome changes. However, no detectable changes were observed in the liver and thymus of both C3H and Swiss newborn mice thus treated.

The mice were also studied for chromosome alteration 20 and 50 days after virus inoculation at birth. None of the mice thus treated showed chromosome changes in the limited number studied. According to Moloney (*32*), the incidence of the viral-induced leukemia was 100%, with a latent period of approximately 2 to 3 months after virus inoculation. It was also reported by Dunn, Moloney, Green, and Arnold (*33*) that 6-week-old mice given the virus showed no pathologic changes until 8 weeks had elapsed, when many megakaryocytes were observed in the bone marrow. The most significant alterations appeared in the thymus of the treated mice. Further experiments must be done regarding the correlation between histopathologic and chromosome changes at the initial stage of leukemia.

In our experiments, the Swiss mice fostered by the virus-infected female or inoculated with the virus at birth developed markedly enlarged thymus, lymph nodes, and spleen late in the disease. Analyses were made on animals in the course of the disease to follow the chromosome changes. Eight of 15 spleens consisted of cells with a predominantly normal diploid chromosome complement, while 7 of 15 spleens had cells predominantly hyperdiploid. Yoshida and Law (*21*) studied a naturally infected Moloney disease in such strains as C3H/Gs, C3H/Fg, C3H/-Lw, BALB/c, and RFM; although normal diploid chromosomes were observed in all cells of 12 C3H/Lw strain mice, hyperdiploidy was found in 2 of 5 animals in C3H/Gs. These results suggest there may be some difference of chromosome number in leukemic cells in different strains. The high frequency of aneuploidy observed in our experiment may be due to a difference in the strain of mice or to the difference in technique.

Some variations in ploidy in different tissues were observed. An interesting finding is that the enlarged thymus in 2 mice with leukemia induced by mother's milk (#9 male and #14 female) showed a diverse distribution of chromosome number, and polyploid cells were more frequent in the thymus than in the spleen and bone marrow cells. These findings seem important in connection with the possible thymic origin in leukemogenesis. Ford, Hamerton, and Mole (*13*) observed that, in a spontaneous leukemia of the AK mouse, the frequency of both diploid cells differed in the bone marrow, spleen, thymus, and other lymph nodes. They suggested that the difference may be attributed to the environment of these organs. In a pathogenesis study of various viruses, Rich and his co-workers (*20*, *22*, *34–36*) observed specific alterations in thymic tissue. Important to our results was the fact that the period of preleukemic changes had already occurred when chromosome alterations were first observed. Thus they concluded that these nonspecific chromosome changes resulted from, rather than caused, the leukemogenic process.

Stich (*37*) and Nadler (*38*) found chromosome changes in DMBA-induced thymic lymphoma in Swiss mice, and X-ray-induced thymic leukemia in C57BL/6J mice. More recently, there were chromosomal alterations (41 chromosomes and an extra marker) in the bone marrow of radiation-induced leukemia in RF mice (*39*). These cytogenetic results in the leukemias induced by carcinogens, X rays, and viruses support the idea that chromosome changes occur in certain types of leukemia, in a particular strain of mice.

The etiologic importance of the thymic tissue in leukemogenesis was reported by Miller (*40*). Lieberman, Haran-Ghera, and Kaplan (*41*) demonstrated that radiation-induced lymphoma virus (RLV) acts directly and locally on the thymus in lymphoma induction. Siegler and Rich (*36*) studied the pathogenesis of a murine radiation lymphoma in C57BL mice. The lymphoma arose in small, contracted thymuses, after alternate lympholysis and regeneration of cortical epithelial cells and not from regenerating thymuses. In many of the virus-induced lymphocytic leukemias, initial cellular changes have occurred in thymic tissue, where submicroscopic changes in the chromosomes may also exist. A detectable chromosome alteration may develop in certain conditions, depending on the nature of the virus, the host, and the cell response.

Ford *et al.* (*13*) Hauschka (*42*), and other investigators regard chromosomal alteration as a phenomenon of primary etiologic significance in the origin and progression of neoplastic growth. Kaplan (*43*), Koller (*44*), and Bayreuther (*19*) consider such karyotypic alterations as a secondary phenomenon, the results of the malignant process rather than the cause. Further cytologic experiments remain to be done on leukemogenesis during the course of the vertical natural transmission of the leukemogenic virus.

A preliminary study on the possibility of metastasis of leukemic cells from pregnant mothers to their offspring has been conducted. The transplanted leukemic cells with 41 chromosomes originating from MLV-induced leukemia in C3H mice were injected into pregnant mice at different days of gestation. Twenty offspring from 5 sets of experiments were studied for chromosome number. No evidence of a possible cellular transmission from mothers to offspring was obtained except once. It seems unlikely that leukemic cells metastasize from the mothers to their fetuses through the placenta. However, strangely enough, most of the liver cells (167/170) of 1 mouse of a certain litter, consisting of 6 newborns whose mother had received intravenously leukemic cells with 41 chromosomes at the 16th day of gestation, showed 41 chromosomes. Hungerford (*45*) reported that chromosome counts from 10-day fetal metaphases were predominantly diploid in Swiss ICR/Ha mice. In our experiment, such high frequency of hyperdiploid cells (41 chromosomes) was not observed in the liver cells either in the control newborn mice, or in the experimental animals. If these cells with 41 chromosomes detected in an offspring are of the same origin as the donor cells, this fact seems to include various problems of a possible congenital leukemia, embryologically and oncologically. However, further studies must be done before any conclusions on the metastasis of leukemic cells from the mothers to their offspring can be made.

SUMMARY

The results of investigations on the vertical transmission of certain murine leukemia viruses reported by Gross, Law, Moloney, and others have been confirmed and extended in this laboratory. During our studies, in 7 of 15 animals chromosomal changes (aneuploidy and aberrations) were noted in the cells of the bone marrow, spleen, and other organs of leukemic mice infected by the virus. A diverse distribution of chromosome numbers (including tetraploids) was also observed in the cells of thymic tissue of mice in the later stages of "naturally" MLV-induced leukemia. These findings may be related to the observation of the thymic origin of virus-induced leukemia in mice. In addition, these studies were extended to the investigation of a possible extraembryonic transmission of the agent in murine animals: (*a*) the liver and thymuses of newborn mice from mothers infected during pregnancy; (*b*) young adult mice, 20 and 50 days post infection; (*c*) for possible chromosome changes, nonleukemic old mice that had a chance to be infected from the parents. No detectable alteration of chromosomes was observed in these animals. The possibility of metastasis of leukemic cells into the fetus from the mother mouse is reported.

REFERENCES

(*1*) Ida, N., Moloney, J. B., and Taylor, H. G.: Vertical transmission of Moloney leukemia virus in A/LN and BALB/c strains of mice. Gann 56: 239–249, 1965.

(*2*) Ida, N., Moloney, J. B., Taylor, H. G., and Trentin, J. J.: Placental transmission of Sarcoma 37 leukemia virus. *In* Res Report Project M 11/p 11. Houston, Univ Texas, M. D. Anderson Hospital and Tumor Institute, 1961, p 226.

(*3*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*4*) Law, L. W.: Influence of foster-nursing on virus-induced and spontaneous leukemia in mice. Proc Soc Exp Biol Med 111: 615–623, 1962.

(*5*) Gross, L.: Studies on pathogenic properties and natural transmission of a mouse leukemia virus. *In* Ciba Foundation Symp on Tumor Viruses of Murine Origin. London, J. & A. Churchill, Ltd., 1962, p 159.

(*6*) Fukuhara, A., Ida, N., and Ohba, Y.: Electronmicroscopic study on Moloney leukemia virus. Acta Haemat Jap 27: 253, 1964.

(*7*) Ida, N., Fukuhara, A., and Ohba, Y.: Vertical transmission of Moloney leukemia virus in Swiss mice. Proc Jap Cancer Assoc, 23d General Meeting: 166–167, 1964.

(*8*) Fukuhara, A., Ida, N., Ohba, Y., Date, Y., Watanabe, S., Nakatani, A., and Takata, H.: Electron microscopic study of Moloney leukemia virus in the process of its vertical transmission. Proc Jap Cancer Assoc, 23d General Meeting: 158–159, 1964.

(*9*) Law, L. W., and Moloney, J. B.: Studies of congenital transmission of a leukemia virus in mice. Proc Soc Exp Biol Med 108: 715–723, 1961.

(*10*) Gross, L.: Vertical transmission of Passage A leukemia virus from inoculated C3H mice to their untreated offspring. Proc Soc Exp Biol Med 107: 90–93, 1961.

(*11*) ———: Transmission of mouse leukemia virus through milk of virus-injected C3H female mice. Proc Soc Exp Biol Med 109: 830–836, 1962.

(*12*) Feldman, D. G., Gross, L., and Dreyfuss, Y.: Electron microscopic study of the Passage A mouse leukemia virus in mammary glands of pregnant, virus-injected, C3H(f) mice. Cancer Res 23: 1604–1607, 1963.

(*13*) Ford, C. E., Hamerton, J. L., and Mole, R. H.: Chromosomal changes in primary and transplanted reticular neoplasms of the mouse. J Cell Comp Physiol 52 (Suppl 1): 235–269, 1958.

(*14*) Stich, H. F., Wakonig, R., and Axelrad, A. A.: Chromosome complements of spontaneous leukemia in AKR mice. Nature (London) 184: 998–999, 1959.

(*15*) Wakonig, R., and Stich, H. F.: Chromosomes in primary and transplanted leukemia of AKR mice. J Nat Cancer Inst 25: 295–305, 1960.

(*16*) Kurita, Y., and Yoshida, T. H.: Chromosomal alteration and the development of tumors. VII. Karyological analysis of spontaneous and induced leukemias in mice. Gann 52: 257–264, 1961.

(*17*) Cailleau, R., and Munro, M. M.: Chromosome patterns in the AKR mouse: Spontaneous leukemia, ascites transfers, and tissue culture cell lines. J Nat Cancer Inst 33: 813–824, 1964.

(*18*) Wakonig-Vaartaja, R.: Chromosomes in leukemias induced by S37 and Friend viruses. Brit J Cancer 15: 120–122, 1960.

(*19*) Bayreuther, K.: Chromosomes in primary neoplastic growth. Nature (London) 186: 6–9, 1960.

(*20*) Tsuchida, R., and Rich, M. A.: Chromosomal aberration in viral leukemogenesis. I. Friend and Rauscher leukemia. J Nat Cancer Inst 33: 33–47, 1964.

(*21*) Yoshida, T. H., and Law, L. W.: Karyological study on mouse leukemias induced by mouse leukemia viruses. Proc Jap Cancer Assoc, 23d General Meeting: 336, 1964.

(*22*) Rich, A., Tsuchida, R., and Siegler, R.: Chromosome aberrations: Their role in the etiology of murine leukemia. Science 146: 252–253, 1964.

(*23*) Fox, M., and Zeiss, I. M.: Chromosome preparation from fresh and cultured tissues using a modification of the drying technique. Nature (London) 192: 1213–1214, 1961.

(*24*) Ingalls, T. H., Ingenito, E. F., and Curley, F. J.: Acquired chromosomal anomalies induced in mice by injection of teratogen in pregnancy. Science 141: 810–812, 1963.

(*25*) Gross, L.: Mouse leukemia: An egg-borne virus disease; with a note on mouse salivary gland carcinoma. Acta Haematol 13: 13–28, 1955.

(*26*) ———: Presence of leukemic agent in normal testes and ovaries of young mice of Ak line. Acta Haematol 10: 18–26, 1953.

(*27*) Dmochowski, L., Grey, C. E., Padgett, F., and Sykes, J. A.: Studies on the structure of the mammary tumor-inducing virus (Bittner) and of leukemia virus (Gross). *In* Viruses, Nucleic Acids and Cancer. Proc 17th Ann Symp on Fundamental Cancer Res, Univ Texas, M. D. Anderson Hospital and Tumor Institute. Baltimore, William & Wilkins Co., 1963.

(*28*) Fekete, E., and Otis, H. K.: Observations on leukemia in AKR mice born from transferred ova and nursed by low-leukemic mothers. Cancer Res 14: 445–447, 1954.

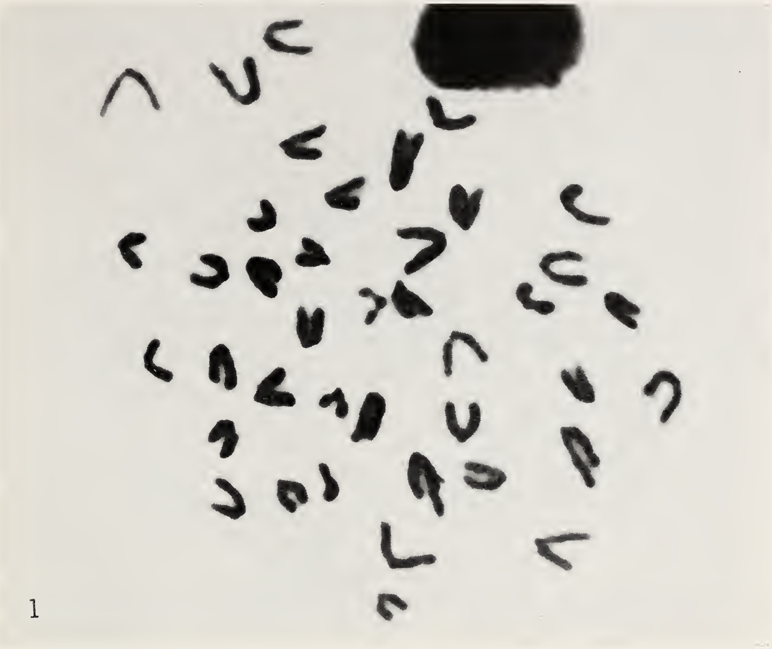
(*29*) Law, L. W.: Studies of experimental transmission of leukemogenic virus infection in mice. J Nat Cancer Inst 34: 543–549, 1965.

(*30*) Mirand, E. A., and Grace, J. T., Jr.: Transmission of Friend virus disease from infected mothers to offspring. Virology 16: 344–347, 1962.

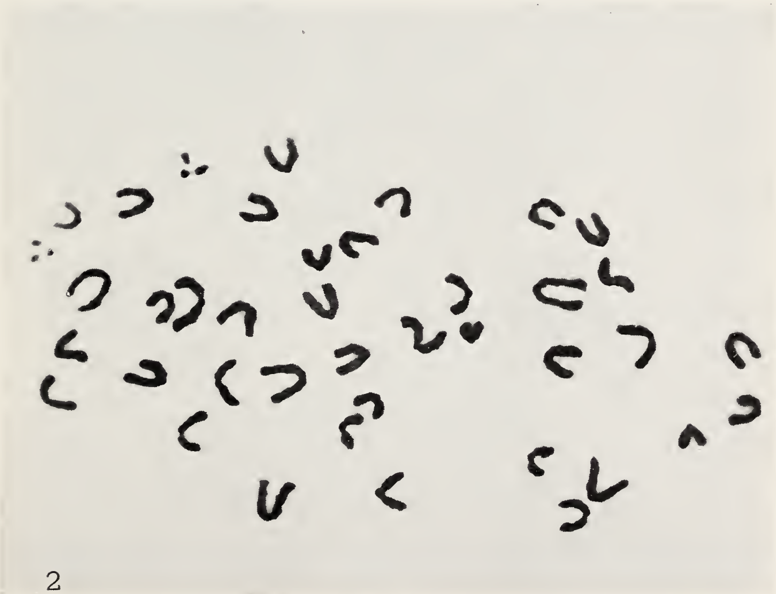
(*31*) Krischke, W., and Graffi, A.: The transmission of the virus of myeloid leukaemia of mice by the milk. Acta Un Int Cancr 19: 360–361, 1963.

(*32*) Moloney, J. B.: Properties of a leukemia virus. Nat Cancer Inst Monogr 4: 7–37, 1960.

(33) Dunn, T. B., Moloney, J. B., Green, A. W., and Arnold, B.: Pathogenesis of a virus-induced leukemia in mice. J Nat Cancer Inst 26: 189–221, 1961.
(34) Siegler, R., and Rich, M. A.: Unilateral histogenesis of AKR thymic lymphoma. Cancer Res 23: 1669–1678, 1963.
(35) Siegler, R., and Rich, M. A.: Comparative pathogenesis of viral lymphoma. Proc Amer Assoc Cancer Res 5: 58, 1964.
(36) ———: Pathogenesis of murine radiation lymphoma. Proc Amer Assoc Cancer Res 6: 59, 1965.
(37) Stich, H. F.: Chromosomes of tumor cells. I. Murine leukemias induced by one or two injections of 7,12-dimethylbenz[*a*]anthracene. J Nat Cancer Inst 25: 649–661, 1960.
(38) Nadler, C. F.: Chromosomal patterns of irradiated leukemic and nonleukemic C57BL/6J mice. J Nat Cancer Inst 30: 923–931, 1963.
(39) Wald, N., Upton, A. C., Jenkins, V. K., and Borges, W. H.: Radiation-induced mouse leukemia: Consistent occurrence of an extra and a marker chromosome. Science 143: 810–813, 1964.
(40) Miller, J. F. A. P.: Etiology and pathogenesis of mouse leukemia. Advances Cancer Res 6: 291–368, 1961.
(41) Lieberman, M., Haran-Ghera, N., and Kaplan, H. S.: Direct action of a leukemogenic virus on the thymus. Proc Amer Assoc Cancer Res 6: 40, 1965.
(42) Hauschka, T. S.: The chromosomes in ontogeny and oncogeny. Cancer Res 21: 957–974, 1961.
(43) Kaplan, H. S.: Carcinogenesis: Mechanisms of Action. Ciba Foundation Sympos (Wolstenholme, G. E. W., ed.), 1959, pp 233–248.
(44) Koller, P. C.: Chromosome behavior in tumors: Readjustments to Boveri's theory. *In* Cell Physiology of Neoplasia. Austin, Texas, Univ Texas Press, 1960, pp 9–48.
(45) Hungerford, D. A.: Chromosome number of 10-day fetal mouse cells. J Morph 97: 497–509, 1955.



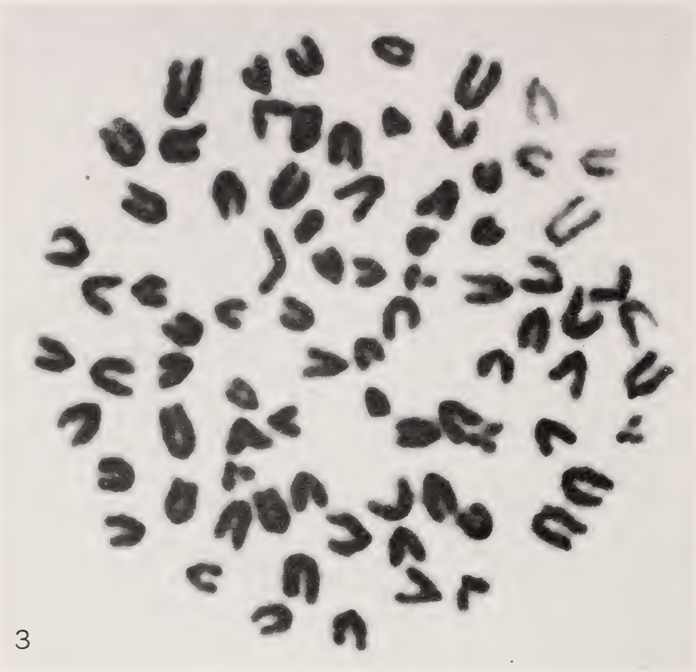
1



2

FIGURE 1.—Metaphase from 1-day-old Swiss mouse whose mother was given Moloney virus during pregnancy. Forty chromosomes are present in thymus. × 1,000

FIGURE 2.—Metaphase from 1-day-old Swiss mouse whose mother was given Moloney virus during pregnancy. Liver showed 40 chromosomes. × 1,000



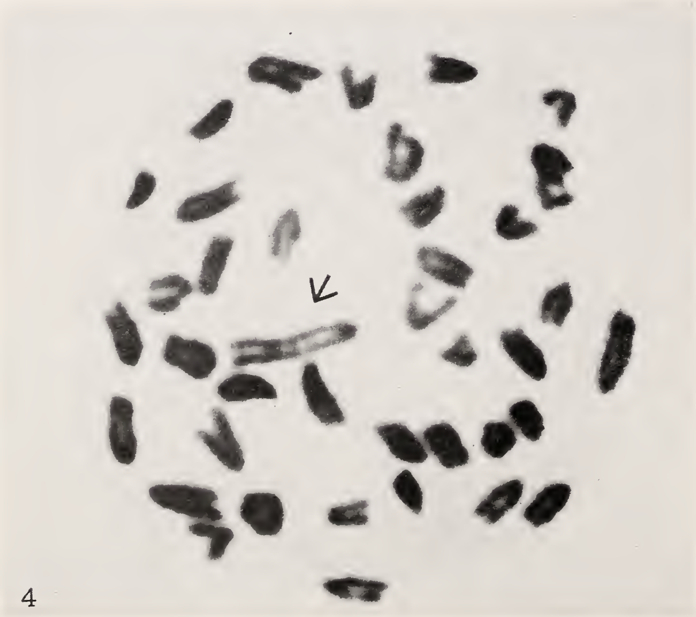
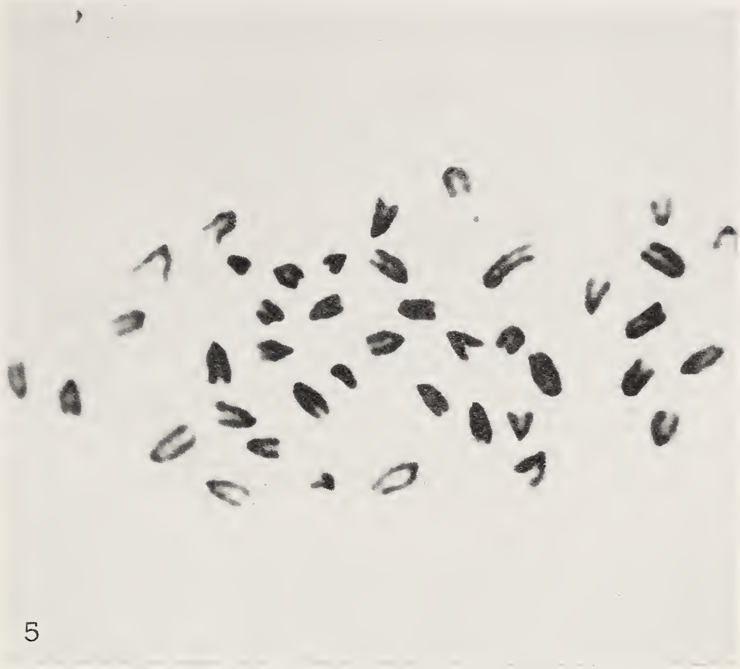


FIGURE 3.—Metaphase from Swiss mouse (#9 male) fostered by virus-infected mother. Latent period was 95 days. Eighty-one chromosomes are present in spleen cell. × 1,000

FIGURE 4.—Metaphase from Swiss mouse (#14 female) fostered by virus-infected mother. Latent period was 274 days. Forty chromosomes are present, including a large chromosome (*arrow*). × 1,000



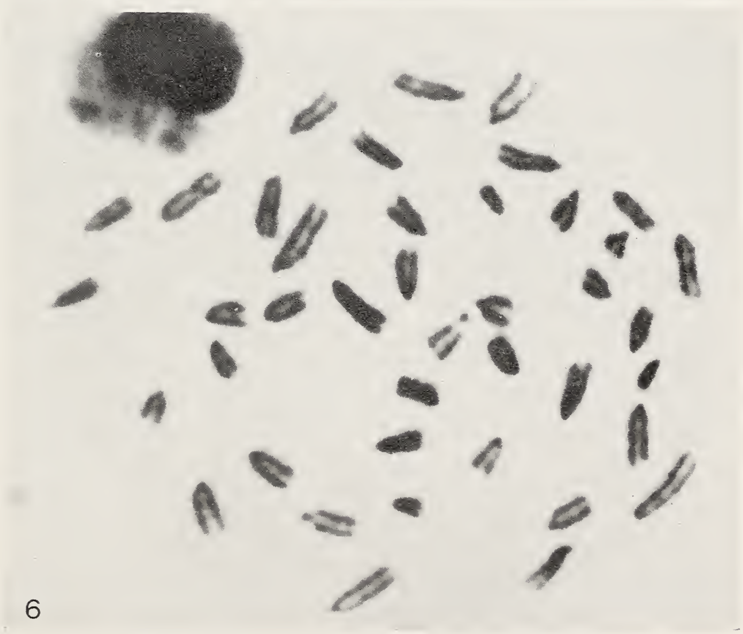


FIGURE 5.—Metaphase from transplanted leukemia in C3H #58 female, showing 41 chromosomes in the cell of ascitic fluid. × 1,000

FIGURE 6.—Metaphase of liver cell from 1-day-old mouse whose mother was given leukemic cells with 41 chromosomes during pregnancy. Most of the liver cells examined showed 41 chromosomes. × 1,000

Studies With Malignant Lymphomas: Possible Interaction Problems Between Chemical and Viral-Inducing Agents [1,2]

Bela Toth *and* Philippe Shubik,[3] *Division of Oncology, The Chicago Medical School, Institute for Medical Research, Chicago, Illinois*

One of the main purposes of this Symposium was to examine the significance of the different inducing factors and their relationship to the development of malignant lymphoma. These include viruses, ionizing radiations, chemical substances, and hormones. It is rather difficult for a single group of investigators to study all these factors simultaneously, since their mechanism of action may vary significantly. Therefore, our investigations during the past 7 years were essentially limited to a group of chemical substances, but we have also been involved to some extent in the more fashionable field of virological leukemia research.

We should like first to point out an important but often overlooked fact in leukemia studies with carcinogenic chemicals: In addition to the induction of malignant lymphomas in the treated animals, a significant number of other types of tumor is usually induced. This observation poses difficult questions: If the malignant lymphoma is basically regarded as a virus-induced neoplasia as shown under various experimental circumstances, what is the cause and explanation of the other induced tumors? Do they belong to the pure chemically induced group? Where can we draw the line in the mechanism of action between the two inducing agents, and does such a dividing line exist at all?

It was known for quite some time that polycyclic hydrocarbons administered to adult mice can induce or enhance leukemia in several strains (*1–6*). Our contribution to this field came when we demonstrated that only a single, relatively low dose of carcinogen given at birth can exert such an effect (*7*). This method proved highly economical and practical, and since then it is widely used in other laboratories (*8–10*).

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] These investigations were supported by Public Health Service grants CS-9212 and CA-4665 from the National Cancer Institute.

[3] We wish to acknowledge our gratitude to Dr. Peter N. Magee for the extraction of DNA and RNA preparations.

Our first report appeared in 1959 showing that 7,12-dimethylbenz[*a*]anthracene (DMBA) injected subcutaneously into newborn Swiss mice produced many malignant lymphomas, stem cell type, and lung adenomas (*7*). We extended this study by using, in addition to DMBA, benzo[*a*]pyrene (BP), 3-methylcholanthrene (MCA), dibenz[*a,h*]anthracene (DBA), and urethan and employing both subcutaneous and intraperitoneal administrations. The results of our second study confirmed those previously found with DMBA and also revealed that tumors were induced with BP, MCA, DBA, and urethan (*11*) at sites other than the lymphoreticular system and lung.

We then investigated the influence of DMBA dosage and the age of the host on tumor development by giving from 4 to 6 different single dose levels to newborn, 2- and 4-week-old Swiss mice (*12*). The results indicated that 1) the newborn Swiss mouse is more sensitive to the leukemogenic action of DMBA than the 2- and 4-week-old mice; 2) most of the DMBA-induced malignant lymphomas belong to the stem (undifferentiated) cell type, whereas in the untreated animals the lymphocytic, histiocytic, or both (mixed) types predominated; 3) the DMBA-induced malignant lymphoma, stem cell type, appeared earlier than the heterogeneous groups of lymphomas that occurred after the age of 35 weeks.

The enhanced susceptibility of the newborn mouse to the induction of lymphomas by chemicals apparently is related to the inability of the animal to "detoxify" certain carcinogens. We have demonstrated that DMBA persists in the newborn mouse for 10 days at a level not seen in the adult after 48 hours (*13*). A similar result was obtained by Berenblum and coworkers with urethan. This observation is pertinent to the nature of the "proximate" carcinogen, but also obviates the necessity for entirely hypothetical explanations, such as the reduced immunological response of the newborn mouse favoring a viral factor.

At this stage in our lymphoma study, hoping to enlarge the significance of the results obtained in Swiss mice, we examined the oncogenic response of AKR mice to DMBA. Expectedly, we observed an accelerated development of malignant lymphomas, starting at 10 weeks of age in mice treated at birth with DMBA, whereas among the controls the lymphomas appeared only after 20 weeks of age (*14*). The incidence of malignant lymphoma was similar in the treated as in the untreated group. In untreated AKR mice the incidence of malignant lymphoma is around 80%, and its development is due to the presence of inherent leukemia virus.

We then performed experiments with golden hamsters and Lewis rats, treated subcutaneously at birth with various single doses of DMBA (*15, 16*) to discover whether their oncogenic response was similar to the response of Swiss mice. The main neoplastic reactions in those two species were limited to the induction of topical tumors: dermal melanocytomas and subcutaneous sarcomas. In addition to the local tumors, a few malignant lymphomas were seen in the treated animals of both species; therefore the evaluation of their significance is rather impossible.

In conclusion, our findings in hamsters and rats regarding the induction of malignant lymphomas by a polycyclic hydrocarbon were practically negative. Since in both species the animals developed topical tumors at the sites of treatment, we were forced to substitute the DMBA with a nonlocally acting leukemogenic chemical, urethan. Presently, an appropriate number of animals of different species treated with urethan are under observation; however, our prospect relating to the induction of lymphoma is not too optimistic.

Our colony of Swiss mice was originally obtained from The Jackson Laboratory, Bar Harbor, Maine, in 1951, and since then bred randomly in our laboratory. The highest incidence of malignant lymphoma in the untreated controls was 16% in females and 4% in males (*17*). Speculation on the biological nature of this tumor in the controls was intriguing. In the meantime, other groups of investigators revealed, with the electron microscope, the presence of characteristic leukemia virus-like particles in the lymphoreticular tissues of these leukemia-bearing mice (*18*, *19*). Soon we were in the middle of this controversy. Considering the limitations of the morphological approach in identifying viral particles, which we do not intend to summarize here, it was more promising to search for biologically active techniques, *i.e.*, the cell-free filtration method successfully employed in viral leukemogenesis by Gross (*20*).

Interestingly, our first pilot experiment, in which the cell-free filtrate was prepared by the Gross method from a DMBA-induced thymic malignant lymphoma in a Swiss mouse, was positive (*21*). There were other successful studies before ours in the cell-free transmission of chemically induced tumors, such as the tar- or 3-methylcholanthrene-induced sarcomas in chickens (*22*, *23*). The findings obtained in radiation-induced leukemia studies are also relevant here: Gross (*24*) and Lieberman and Kaplan (*25*) independently demonstrated the cell-free transmissibility of X-ray-induced leukemias in mice. But a series of efforts to transmit chemically induced lymphomas by cell-free filtrates were basically negative (*26*, *27*). Recently, several contributions have aroused considerable interest, and the subject was reopened for further consideration. The 3-methylcholanthrene-induced mouse lymphoma, the 4-nitroquinoline-*N*-oxide-induced mouse lymphoma, the 3-methylcholanthrene-induced newt malignant melanoma, and the DMBA-induced rat breast adenocarcinoma were successfully transmitted by cell-free filtrates (*28–31*).

These various, often isolated results prompted us to reinvestigate the problem using larger groups of animals, including several controls. Swiss mice from our laboratory were used, and the cell-free filtration method of Gross was employed as follows: Fresh thymus, spleen, and lymph nodes from mice killed by ether were suspended in physiological saline 1:5 at 0° C. The tissues were homogenized until a fine suspension was obtained (1–2 min). The cell suspension was centrifuged (model PR–2, International Equipment Co., Boston, Mass.) at 3000 rpm (1400

× g) for 15 minutes at 0° C. The supernatant was centrifuged (model L preparative ultracentrifuge, Beckman Instruments, Inc., Belmont, Calif.) at 9500 rpm (7000 × g) for 6 to 8 minutes at 0° C. Then the second supernatant was passed through a Selas, porosity 02, porcelain filter candle, a new one used at each occasion.

Five groups of mice comprised the experiment and, in each group, the mice were inoculated subcutaneously on the back at birth (less than 24 hours old) with 0.05 ml cell-free filtrates in physiological saline. The treatment, source of materials, number of treated animals, and the survival rate, summarized in table 1, were as follows:

> Group 1: The cell-free filtrate was obtained from 5 untreated, control, 8-week-old female mice and the material was injected into 38 mice.
>
> Group 2: The cell-free filtrate was prepared from an untreated 65-week-old female mouse that had malignant lymphoma, lymphocytic type, and the material was injected into 50 mice.
>
> Groups 3, 4, and 5: The cell-free filtrates were obtained from three, 10-, 13-, and 16-week-old female mice in which the malignant lymphomas, stem cell type, were induced by a single injection of 100 or 1000 μg DMBA given at birth or at 4 weeks. The cell-free filtrate of each mouse represented a separate group and each filtrate was injected into 55, 43, and 29 mice.

The animals were observed for their lifespan (table 1). At death all were autopsied and histological sections were routinely used. The number, incidence, and latent period of malignant lymphomas and other tumors are shown in table 2. It is evident from the figures that the cell-free filtrates obtained from the control, "spontaneous" lymphoma-bearing mouse, and those from DMBA-induced lymphomas failed to exert leukemogenic action in the various groups.

Our present "reinvestigation" is somewhat discouraging, but these negative results do not contradict other successful cell-free filtration findings, since there was probably a living, changeable factor in an organism whose nature is not comparable to the constant action of certain chemicals.

Apparently, the transmission by cell-free filtrates of chemically induced malignant lymphomas or other tumors can be interpreted various ways. It is conceivable that an activation of viral or infectious agents was obtained by the chemical, and it is also equally possible that with the filtration an infectious material was accidentally picked up which lay dormant and was harmless in the original host and was transferred to the new host where it acquired its tumorigenic potency under favorable conditions.

Parallel to the cell-free filtration studies, we conducted a series of experiments concerning mainly the possible tumorigenic capability of various nucleic acids. Because we knew that oncogenic viruses are composed of a central core of either DNA or RNA, we considered the possibility of

TABLE 1.—Studies with cell-free filtrates in Swiss mice

Group	Subcutaneous injection of cell-free filtrate*	Source of treatment (organs)†	Number of animals treated at birth	Number of survivors at weaning	Number of survivors after weaning (age in weeks)													
					10	20	30	40	50	60	70	80	90	100	110	120	130	140
1	In 0.05 ml of physiological saline	5 untreated mice (8 weeks old)	38	10 ♀ 23 ♂	9 23	8 23	8 22	8 21	7 19	7 17	6 14	4 10	4 2	1 1	1 1	1 —	1 —	— —
2	In 0.05 ml of physiological saline	Untreated mouse (65 weeks old) with "spontaneous" lymphoma	50	26 ♀ 19 ♂	27 18	26 18	24 17	23 17	21 14	20 10	12 7	10 3	8 1	6 —	1 —	— —	— —	— —
3	In 0.05 ml of physiological saline	Mouse lymphoma (13 weeks old) induced by 100 μg DMBA given at birth	55	21 ♀ 17 ♂	21 17	20 16	20 13	20 12	20 10	17 8	14 5	10 3	8 1	3 —	1 —	— —	— —	— —
4	In 0.05 ml of physiological saline	Mouse lymphoma (16 weeks old) induced by 1000 μg DMBA given at 4 weeks of age	43	13 ♀ 22 ♂	13 22	13 22	13 20	13 20	13 19	13 16	12 15	8 13	7 1	5 —	— —	— —	— —	— —
5	In 0.05 ml of physiological saline	Mouse lymphoma (10 weeks old) induced by 100 μg DMBA given at birth	29	12 ♀ 16 ♂	12 13	12 13	11 13	9 13	9‡ 12‡									

*The cell-free filtrate (1:5) was prepared by the Gross method.
†Thymus, spleen, and lymph nodes.
‡Some of the animals are still alive.

TABLE 2.—Incidence of tumors in animals treated at birth with cell-free filtrates

Group	Effective No. of animals	Number of animals with:		
1	10 ♀	—		
	23 ♂	2 malignant lymphomas, lymphocytic (45, 59)* 1 malignant lymphoma, histiocytic (84) 1 malignant lymphoma, unclassifiable (55)	2 lung adenomas (59, 83) 2 hemangiomas of liver (78, 82) 1 hemangioma of kidney (115)	1 subcutaneous fibrosarcoma (87) 1 papilloma of skin (87)
2	26 ♀	3 malignant lymphomas, lymphocytic (42, 65, 65) 1 lung adenoma (76) 1 lung adenocarcinoma (66)		
	19 ♂	2 malignant lymphomas, lymphocytic (42, 55) 1 malignant lymphoma, histiocytic (59)	1 hemangioma of liver (88)	
3	21 ♀	1 malignant lymphoma, histiocytic (112) 2 lung adenomas (90, 94) 1 hemangioma of ovary (78)	1 adenocarcinoma of breast (61) 1 hemangiosarcoma of liver (58) 1 hemangioma of liver (94)	
	17 ♂	1 malignant lymphoma, stem cell (28) 1 malignant lymphoma, lymphocytic (33) 1 lung adenoma (95)		
4	13 ♀	4 malignant lymphomas, histiocytic (64, 72, 78, 80) 1 lung adenoma (96) 1 adenocarcinoma of breast (96)		
	22 ♂	1 malignant lymphoma, lymphocytic (51) 1 malignant lymphoma, histiocytic (80) 3 lung adenomas (51, 83, 87)	1 hemangiosarcoma of liver (49)	
5	12 ♀	1 malignant lymphoma, lymphocytic (28)		
	16 ♂	1 malignant lymphoma, lymphocytic (41)		

*Numbers in parentheses are latent periods in weeks.

extracting the nucleic acids hoping they might transmit the tumorigenic agent. As an alternative, we thought that the DMBA might possibly alter the nucleic acid bases—changes which might lead to the development of neoplasia. Finally, tumorigenic properties of the nucleic acid preparations isolated from various sources were reported (*32–35*). The experiment was so devised that DNA and RNA were isolated from untreated animals serving as controls, from DMBA-treated mice which might indicate a stage of reversible alteration, and from mice with tumors, intended to serve as the main group in which the probable alteration caused by the chemical reached a definite irreversible state. Consideration should be given to the nature of extraction methods, known to be harsh, since one of the chief reagents is phenol, and workers in this field claim that the methods used destroy the living cell, including the possibly present virus. The extraction methods for the isolation of biologically active DNA and RNA were Kirby's (*36*, *37*).

The DNA was extracted by homogenization of fresh tissues in 12 volumes of *p*-aminosalycilate. The homogenate was mixed with an equal volume of 90% phenol and shaken for 1 hour. The mixture was centrifuged (model PR-2, International Equipment Co.) at about 5000 rpm for 1 hour at 0° C, and the pale-yellow supernatant was removed. It was then precipitated with an equal volume of 2-ethoxyethanol. The precipitate (DNA) was finally removed with a glass rod and dissolved in a small volume of water (1–2 ml).

The RNA was extracted by homogenization of fresh tissues in 8 volumes of 0.5% naphthalenedisulfonic acid (*p*H 6.6). Eight volumes of Kirby's phenol reagent (90% aqueous phenol contains 0.5% 8-hydroxyquinoline) were added to the homogenate and this mixture was shaken for 15 minutes. Later it was centrifuged (model PR-2, International Equipment Co.) at 2000 rpm for 30 minutes at 0° C followed by precipitation with 2 volumes of ethanol. The precipitate (RNA) was dissolved in physiological saline for the desired concentration for injection. When we extracted the RNA from the carcinogen-treated animals, it was purified with ethanol, ether, and ether washings.

The DNA and RNA treatments, their sources, the number of treated animals, and their survival rates are shown in table 3. Swiss mice were used and the extracts were injected subcutaneously in 0.05 ml physiological saline. The groups are briefly described as follows:

Group 1: The DNA was extracted from the thymuses of 17 newborn untreated mice and was divided and given to 20 mice.

Group 2: The RNA extract was obtained from the thymuses of 23 newborn untreated mice, and approximately 100 μg RNA was injected into each of 28 mice.

Group 3: The DNA was extracted from the thymuses of three 8-week-old mice inoculated subcutaneously at birth with 100 μg DMBA in tricaprylin oil. Approximately 250 μg DNA was injected into each of 17 mice.

TABLE 3.—Studies with DNA and RNA extracts* in Swiss mice

Group	Treatment (sc injection)	Source of treatment	Number of animals treated at birth	Number of survivors at weaning	Number of survivors after weaning (age in weeks)												
					10	20	30	40	50	60	70	80	90	100	110	120	130
1	DNA extract in 0.05 ml physiological saline	17 newborn mice, untreated thymuses	20	7♀	7	7	7	6	6	6	5	3	3	—	—	—	—
				10♂	10	8	8	8	8	8	5	4	—	—	—	—	—
2	RNA extract 100 µg in 0.05 ml physiological saline	23 newborn mice, untreated thymuses	28	8♀	8	8	8	8	8	8	7	2	2	1	1	1	—
				20♂	17	17	17	14	13	11	7	3	2	—	—	—	—
3	DNA extract 250 µg in 0.05 ml physiological saline	Thymuses from 3 mice (8 weeks old) treated at birth with 100 µg DMBA	17	5♀	5	5	5	5	5	4	4	4	—	—	—	—	—
				8♂	8	8	8	8	7	6	5	4	—	—	—	—	—
4	RNA extract in 0.05 ml physiological saline	Thymuses from 3 mice (7–8 weeks old) treated at birth with 100 µg DMBA	16	6♀	6	6	6	6	6	6	6	4	2	1	1	—	—
				9♂	9	9	9	8	8	6	5	3	1	1	1	—	—
5	DNA extract in 0.05 ml physiological saline	Thymic malignant lymphoma induced by an injection of 100 µg DMBA (12-week-old mouse)	62	18♀	17	17	17	16	16	12	8	4	2	—	—	—	—
				17♂	15	15	14	14	14	12	10	10	7	—	—	—	—
6	RNA extract in 0.05 ml physiological saline	Thymic malignant lymphoma induced by an injection of 100 µg DMBA (12-week-old mouse)	65	26♀	25	25	25	25	23	22	19	15	9	1	—	—	—
				27♂	26	26	26	25	22	17	12	6	4	2	—	—	—

*The extracts were prepared by Kirby's method.

Group 4: The RNA was isolated from the thymuses of three 7- and 8-week-old mice inoculated subcutaneously at birth with 100 μg DMBA in tricaprylin oil. The RNA extract was divided and injected into 16 mice.

Group 5: The DNA was extracted from a malignant lymphoma, stem cell type, of the thymus. This tumor was induced in a 12-week-old mouse inoculated subcutaneously at birth with 100 μg DMBA in tricaprylin oil. The extract was divided and injected into 62 mice.

Group 6: The RNA was extracted from a thymic malignant lymphoma, stem cell type, induced in a 12-week-old mouse given a subcutaneous injection at birth of 100 μg DMBA in tricaprylin oil. The extract was divided and injected into 65 mice.

Table 4 shows the number, incidence, and latent periods of all observed tumors in the various groups. The treatments with DNA and RNA extracts resulted in no detectable carcinogenic effect in the inoculated animals. Again our findings gave negative results and because of the limited number of successes obtained by others, we hope that the search for tumorigenic nucleic acids will not be abandoned completely.

In the next series of studies we examined the neoplastic response of AKR mice to two well-known chemical carcinogens, benzo[*a*]pyrene (BP) and dimethylnitrosamine (DMN). There is a basic difference in the carcinogenic effects of these two chemicals. BP is a known agent in inducing or enhancing malignant lymphomas, in addition to inducing other types of tumors (*11*), whereas with DMN no leukemogenic action has yet been observed under a variety of experimental conditions (*38*, *39*). Therefore, we believed it ideal to bring the action of each of the two carcinogenic chemicals to AKR mice in which the well-documented leukemogenic virus is present from birth.

The experiment was divided into the following groups: (*a*) A single dose of 30 μg DMN was injected subcutaneously at birth into each of 74 newborn mice. (*b*) A single dose of 200 μg BP was injected subcutaneously into each of 57 newborn mice. (*c*) Forty-six females and 59 males were untreated.

The results showed that in BP-treated mice a significant number of malignant lymphomas appeared earlier than in the untreated controls, whereas in the DMN-treated groups many pulmonary and hepatic tumors were induced and concomitantly the appearance of malignant lymphomas was unaltered by the treatment.

Our studies with AKR mice showed that malignant lymphomas in the BP-treated mice appear earlier than in the controls. This phenomenon may be an "activation of a neoplastic process" caused ultimately by an oncogenic virus. But it is equally possible that these early malignant lymphomas occur as true induction by the chemical itself without any viral interference. This latter possibility is more conceivable to those who emphasize the capability of BP to induce other tumors, such as sub-

TABLE 4.—Incidence of tumors in Swiss mice treated at birth with DNA and RNA extracts

Group	Effective No. of animals	Number of animals with:
1	7 ♀	1 hepatoma (96)* 1 lung adenoma (99)
	10 ♂	1 hemangioma of liver (61) 3 lung adenomas (76, 81, 87)
2	8 ♀	1 adenocarcinoma of breast (72) 1 lung adenoma (72) 1 adenocarcinoma of ovary (121) 1 malignant lymphoma, histiocytic (76)
	20 ♂	1 lung adenoma (67) 1 hemangioma of liver (53) 1 subcutaneous sarcoma (76) 2 malignant lymphomas, histiocytic (61, 71)
3	5 ♀	—
	8 ♂	2 lung adenomas (75, 89) 1 malignant lymphoma, lymphocytic (65) 1 adenocarcinoma of anal gland (75)
4	6 ♀	3 lung adenomas (74, 94, 115) 1 hemangioma of uterus (94) 1 squamous cell carcinoma of ear (85) 1 malignant lymphoma, histiocytic (85) 2 adenocarcinomas of breast (85, 94)
	9 ♂	2 lung adenomas (53, 66) 1 malignant lymphoma, histiocytic (71)

5	18 ♀	6 adenocarcinomas of breast (57, 78, 78, 80, 85, 92) 2 hemangiomas of ovary (61, 92) 1 adenocarcinoma of uterus (57)	1 squamous cell carcinoma of ear (78) 1 malignant lymphoma, lymphocytic (39) 1 malignant lymphoma, histiocytic (78)
	17 ♂	1 lung adenoma (80) 1 liver cell carcinoma (80) 1 papilloma of skin (91) 1 hemangiosarcoma of pancreas (69)	
6	26 ♀	1 malignant lymphoma, lymphocytic (47) 3 malignant lymphomas, histiocytic (73, 79, 96) 2 lung adenomas (47, 82) 2 hemangiomas of liver (47, 90)	1 hemangioma of ovary (87) 4 adenocarcinomas of breast (63, 86, 105, 106) 1 squamous cell carcinoma of skin (106)
	27 ♂	4 lung adenomas (59, 61, 94, 107) 3 malignant lymphomas, histiocytic (59, 72, 77) 1 hepatoma (63) 1 squamous cell carcinoma of skin (94)	1 hemangioma of liver (60)

*Numbers in parentheses are latent periods in weeks.

cutaneous sarcoma and lung neoplasms, in which viral interference seems illusory. However, one could assume an extremist theoretical viewpoint of the existence of a pluripotent oncogenic agent possibly present in the animals. Such an agent could conceivably, under special experimental conditions, become leukemogenic and/or produce tumors outside the lymphoreticular system. Unfortunately, our experimental devices are inadequate to distinguish the possible different causative mechanisms.

The mode of action of DMN is obviously due to the chemical or to the by-product itself. There is no basis for speculation on the viral interference in DMN carcinogenesis. When one considers the many pulmonary and hepatic tumors induced in the DMN-inoculated animals, and simultaneously takes into account the appearance of malignant lymphomas in this group in which their manifestation is apparently due to the leukemia virus, it then becomes obvious that the carcinogenic chemical, DMN, has not influenced the neoplastic process caused by the oncogenic virus.

Thus the present study provides an example in which two distinct oncogenic agents, dimethylnitrosamine and the leukemia virus, carried out their own separate tumorigenic effects without molesting the neoplastic pathway of the other agent. It seems reasonable that the present results are a negative answer to those virologists who claim that the actions of carcinogenic chemicals can be interpreted only as an alterating effect on the mode of action of a pluripotent oncogenic virus.

Several previous observations suggest that viruses and carcinogenic chemicals act additively in inducing cancer. Rous and Kidd demonstrated that when Shope papilloma virus was injected intravenously into rabbits whose ears had been painted with tar, warts and many metastasizing epitheliomas arose. Tarring alone and the injection of Shope papilloma virus by itself caused a few warts or were ineffective (*40*, *41*). Duran-Reynals reported a higher incidence of tumors in mice, when the vaccinia virus was combined with methycholanthrene, than in mice given MCA alone (*42*). Later Tanaka and Southam (*43*) demonstrated a greater incidence of papillomas, when the West Nile or herpes simplex virus was given during the administration of MCA or BP, than was obtained by either carcinogenic chemical.

Other observations seem more complex than those already mentioned. Recently, the potentiation of viral leukemogenesis in C57BL mice by X irradiation or urethan was reported by Kaplan's group (*44*). The incidence of malignant lymphoma was strikingly enhanced when C57BL mice with the radiation-induced lymphoma virus, were exposed to X ray, to urethan, and to both. The neoplastic response to combined treatment exceeded the simple additive effect and therefore the result suggests a synergistic action between radiation-induced lymphoma virus and X ray plus urethan.

To continue with examples and to add to the complexity, we should consider the similar but independent approach of Berenblum and Trainin (*45*). They observed a significant increase in leukemia in C57BL mice receiving repeated doses of urethan after total-body irradiation, but not when the procedure was carried out in reverse. As a second step in their study, they obtained leukemia when urethan was given to nonirradiated animals previously implanted with tissues from irradiated isogenic donors (*46*). On this basis, they suggested the possibility that urethan acts as a coleukemogenic agent by converting the "transmissible factor" appearing in a precursor stage as an inactive virus into an active leukemogenic one.

In conclusion, considering our various results and including other findings in this field, we believe that there are various examples in which the carcinogenic chemicals and oncogenic viruses can exert their neoplastic actions. Under certain experimental conditions, their oncogenic effects seem distinct and clearly separated. There are other well-documented instances where the chemical substances and viruses, not necessarily oncogenic viral agents, apparently act synergistically, additively enlarging the neoplastic effect of each other. In view of only the present knowledge in experimental leukemogenesis, the situation seems more complicated. With the introduction of highly leukemic inbred strains of mice, the explanation of all results obtained by extrinsic agents, such as carcinogenic chemicals, is somehow influenced, if not made one-sided, by the virus theory. The often-mentioned "virus activation" theory in leukemogenesis, which must be supplemented by proved facts, remains a possible goal for achievement. Because of this interesting and unusual situation, it would seem promising to conduct more experiments with proper observations rather than to meditate hypothetically.

SUMMARY

Experiments mainly with newborn animals, on the induction of malignant lymphomas by various carcinogenic chemicals, were described. They included Swiss and AKR mice, golden hamsters, and Lewis rats. The mice gave positive and the other species, negative results.

Repeated attempts were made to transmit the Swiss mouse lymphoma by cell-free filtrates and nucleic acid extracts. Except for the first pilot study in which the chemically induced lymphoma was successfully transmitted by cell-free filtrates, the repetitions of this work and the DNA and RNA studies were essentially negative.

The possible synergistic, activating, and independent actions of certain carcinogenic chemicals such as 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, and dimethylnitrosamine on the development of virus-induced malignant lymphoma (in AKR mice) were tested and the results discussed.

REFERENCES

(*1*) Kirschbaum, A., Strong, L. C., and Gardner, W. U.: Influence of methylcholanthrene on age incidence of leukemia in several strains of mice. Proc Soc Exp Biol Med 45: 287–289, 1940.

(*2*) Law, L. W.: The induction of leukemia in mice following percutaneous application of 9,10-dimethyl-1,2-benzanthracene. Cancer Res 1: 564–571, 1941.

(*3*) Engelbreth-Holm, J., and Lefèvre, H.: Acceleration of the development of the leukemias and mammary carcinomas in mice by 9,10-dimethyl-1,2-benzanthracene. Cancer Res 1: 102–108, 1941.

(*4*) Furth, J., and Barnes, W. A.: Differences between malignant blood cells from induced and spontaneous leukemias of mice. Cancer Res 1: 17–22, 1941.

(*5*) Morton, J. J., and Mider, G. B.: Some effects of carcinogenic agents on mice subject to spontaneous leukoses. Cancer Res 1: 95–98, 1941.

(*6*) Rask-Nielsen, R.: Local and remote tumours in strain Street mice following injection of large doses of four different carcinogenic hydrocarbons. Brit J Cancer 4: 124–132, 1950.

(*7*) Pietra, G., Spencer, K., and Shubik, P.: Response of newly born mice to a chemical carcinogen. Nature (London) 183: 1689, 1959.

(*8*) Stich, H. F.: Chromosomes of tumor cells. I. Murine leukemias induced by one or two injections of 7,12-dimethylbenz[*a*]anthracene. J Nat Cancer Inst 25: 649–661, 1960.

(*9*) Kelly, M. G., and O'Gara, R. W.: Induction of tumors in newborn mice with dibenz[*a,h*]anthracene and 3-methylcholanthrene. J Nat Cancer Inst 26: 651–679, 1961.

(*10*) Roe, F. J., Rowson, K. E., and Salaman, M. H.: Tumours of many sites induced by injection of chemical carcinogens into newborn mice, a sensitive test for carcinogenesis: the implications for certain immunological theories. Brit J Cancer 15: 515–530, 1961.

(*11*) Pietra, G., Rappaport, H., and Shubik, P.: The effects of carcinogenic chemicals in newborn mice. Cancer 14: 308–317, 1961.

(*12*) Toth, B., Rappaport, H., and Shubik, P.: Influence of dose and age on the induction of malignant lymphomas and other tumors by 7,12-dimethylbenz[*a*]anthracene in Swiss mice. J Nat Cancer Inst 30: 723–741, 1963.

(*13*) Domsky, I. I., Lijinsky, W., Spencer, K., and Shubik, P.: Rate of metabolism of 9,10-dimethyl-1,2-benzanthracene in newborn and adult mice. Proc Soc Exp Biol Med 113: 110–112, 1963.

(*14*) Toth, B., Rappaport, H., and Shubik, P.: Accelerated development of malignant lymphomas in AKR mice injected at birth with 7,12-dimethylbenz[*a*]anthracene. Proc Soc Exp Biol Med 110: 881–884, 1962.

(*15*) Lee, K. Y., Toth, B., and Shubik, P.: Carcinogenic response of the Syrian golden hamster treated at birth with 7,12-dimethylbenz[*a*]anthracene. Proc Soc Exp Biol Med 114: 579–582, 1963.

(*16*) Toth, B., and Shubik, P.: Carcinogenesis in Lewis rats injected at birth with 7,12-dimethylbenz[*a*]anthracene. Brit J Cancer 17: 540–545, 1963.

(*17*) Toth, B., Della Porta, G., and Shubik, P.: The occurrence of malignant lymphomas in urethan-treated Swiss mice. Brit J Cancer 15: 322–326, 1961.

(*18*) de Harven, E., and Friend, C.: Electron microscopy of mouse leukemia viruses. *In* Proc Eur Reg Conf on Electron Microscopy. Delft 2: 990–994, 1960.

(*19*) de Harven, E.: Virus particles in the thymus of the conventional and germfree mice. J Exp Med 120: 857–868, 1964.

(*20*) Gross, L.: Oncogenic Viruses. New York, Pergamon Press, 1961.

(*21*) Toth, B.: Development of malignant lymphomas by cell-free filtrates prepared from a chemically induced mouse lymphoma. Proc Soc Exp Biol Med 112: 873–875, 1963.

(*22*) McIntosh, J., and Selbie, F. R.: Further observations on filterable tumours induced in fowl by injection of tar. Brit J Exp Path 20: 49–63, 1939.

(*23*) Oberling, C., and Guerin, M.: Sarcome de la poule par methylcholanthrène devenu filtrable. Bull du Cancer 37: 5–14, 1950.

(*24*) Gross, L.: Serial cell-free passage of a radiation-activated mouse leukemia agent. Proc Soc Exp Biol Med 100: 102–105, 1959.

(*25*) Lieberman, M., and Kaplan, H. S.: Leukemogenic activity of filtrates from radiation-induced lymphoid tumors of mice. Science 130: 387–388, 1959.

(*26*) Salaman, M. H.: The early development of tumours in mice inoculated with a cell-free filtrate of mouse leukaemic tissue. Brit J Cancer 13: 76–86, 1959.

(*27*) Latarjet, R.: Viruses in relation to other cancerogenic agents. Cancer Res 20: 807–815, 1960.

(*28*) Irino, S., Ota, Z., Sezaki, T., Suzaki, M., and Hiraki, K.: Cell-free transmission of 20-methylcholanthrene-induced RF mouse leukemia and electron microscopic demonstration of virus particles in its leukemic tissue. Gann 54: 225–237, 1963.

(*29*) Tanaka, T., Kakefuda, T., and Kinosita, R.: Experimental carcinogenesis by 4-nitro-quinoline-N-oxide (4NQO). Proc Amer Assoc Cancer Res 4: 67, 1963.

(*30*) Zavanella, T.: Cell-free transmission of a methylcholanthrene-induced malignant melanoma in amphibians. Tumori 50: 485–498, 1964.

(*31*) Scholler, J., Bergs, V. V., and Groupé, V.: Production of mammary carcinomas in female Sprague Dawley rats by cell free materials derived from mammary carcinomas induced in rats by 7,12-dimethylbenzanthracene (DMBA). Proc Amer Assoc Cancer Res 5: 56, 1964.

(*32*) Lacour, F., Lacour, J., Harel, J., and Huppert, J.: Transplantable malignant tumors in mice induced by preparations containing ribonucleic acid extracted from human and mouse tumors. J Nat Cancer Inst 24: 301–327, 1960.

(*33*) Ito I., and Evans, A. C.: Induction of tumors in domestic rabbits with nucleic acid preparations from partially purified Shope papilloma virus and from extracts of the papillomas of domestic and cottontail rabbits. J Exp Med 114: 485–500, 1961.

(*34*) Hays, E. F., and Carr, A. J.: Oncogenic properties of deoxyribonucleic acid isolated from parotid gland tumors. Cancer Res 22: 1319–1322, 1962.

(*35*) Hays, E. F.: Transmission of mouse leukaemia with purified nucleic acid preparations. Nature (London) 192: 230–232, 1961.

(*36*) Kirby, K. S.: A new method for the isolation of ribonucleic acids from mammalian tissues. Biochem J 64: 405–408, 1956.

(*37*) ———: A new method for the isolation of deoxyribonucleic acids: evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem J 66: 495–504, 1957.

(*38*) Magee, P. N., and Barnes, I. M.: The production of malignant primary hepatic tumours in rats by feeding dimethylnitrosamine. Brit J. Cancer 10: 114–122, 1956.

(*39*) Toth, B., Magee, P. N., and Shubik, P.: Carcinogenesis study with dimethylnitrosamine administered orally to adult and subcutaneously to newborn BALB/c mice. Cancer Res 24: 1712–1722, 1964.

(*40*) Rous, P., and Kidd, J.: The carcinogenic effect of a papilloma virus on the tarred skin of rabbits. I. Description of the phenomenon. J Exp Med 67: 399–428, 1938.

(*41*) Kidd, J., and Rous, P.: The carcinogenic effect of a papilloma virus on the tarred skin of rabbits. II. Major factors determining the phenomenon: the manifold effects of tarring. J Exp Med 68: 529–562, 1938.

MURINE LEUKEMIA

(*42*) Duran-Reynals, F.: Preliminary studies on the development of neoplasia in the skin of mice painted with methylcholanthrene and injected with cortisone and vaccine virus. Ann NY Acad Sci 68: 430–440, 1957.

(*43*) Tanaka, S., and Southam, C. M.: Joint action of herpes simplex virus and 3-methylcholanthrene in production of papillomas in mice. J Nat Cancer Inst 34: 441–451, 1965.

(*44*) Lieberman, M., Haran-Ghera, N., and Kaplan, H. S.: Potentiation of virus leukaemogenesis in C57BL mice by X-irradiation or urethane. Nature (London) 203: 420–422, 1964.

(*45*) Berenblum, I., and Trainin, N.: Possible two-stage mechanism in experimental leukemogenesis. Science 132: 40–41, 1960.

(*46*) ———: Leukemogenesis by urethan in C57BL mice bearing isologous tissues from X-irradiated mice. Science 134: 2045–2047, 1961.

DISCUSSION

Dr. Chieco-Bianchi: I wish to talk about some results obtained in our laboratory which confirm Dr. Toth's data. In AKR mice we injected 25 μg dimethylnitrosamine at birth or 50 μg at 10 days of age. These animals showed no acceleration of leukemia but at advanced age they developed numerous lung and liver tumors. This means that not all chemical carcinogens, but only those having leukemogenic properties, can induce acceleration of leukemia when injected at birth in AKR mice. On the other hand, estrogen, despite its leukemogenic activity, did not induce early development of leukemia when injected in newborn AKR mice.

Dr. Duran-Reynals: I would like to emphasize that chemical carcinogens enhance viral infection, not because they change the virus but because they change the host. At least this seems to have been confirmed by many observations.

Dr. Sinkovics: I would like to ask whether anybody induced Friend- or Rauscher-type erythroblastic leukemias with chemicals.

Dr. Salaman: No, I haven't. Urethan, for example, injected into baby mice, produces exclusively the malignant lymphoma type. But if you take filtrates of such lymphomas and pass them serially, in the way Dr. Gross and others have taught us to do, you get, at least in my experience, types of leukemia varying from the lymphatic through various kinds of reticulum cell and histocyte, to the myeloid type.

What the significance of this is I can't tell you. I have no guarantee, as I believe there is no guarantee in any of these cases, that this agent is either present in the original tumor or that it is inductive or partially inductive of it.

Dr. Furth: As to "carcinogens" other than radiation-activating latent virus, I should like to cite the recent experiments in our laboratory (Proc. Soc. Exp. Biol. Med. 119: 1211, 1965) on estrogen-induced lymphoma in RF mice. One of three estrogen-induced lymphomas was assayed; only one yielded a virus.

The core of the question is whether carcinogens can also induce leukemia by virtue of direct effect and merely yield a carrier virus. This is the core of the problem which, to my knowledge, though tackled with chickens and mammals is still wide open. Cancer cells are good media for viral proliferation.

Dr. Chieco-Bianchi: I think it is relevant to mention here the recent experiments of the De Maeyers on the inhibition of interferon production by carcinogens *in vitro*. They showed that carcinogenic hydrocarbons, but not noncarcinogenic ones, can inhibit the interferon synthesis in cultures (J. Nat. Cancer Inst. 34: 265–276, 1965). Urethan also has a low but clearly detectable inhibiting effect.

Observations on Viral, Chemical, and Radiation-Induced Myeloid and Lymphoid Leukemias in RF Mice[1,2]

A. C. Upton, V. K. Jenkins, H. E. Walburg, Jr., R. L. Tyndall, J. W. Conklin, *and* N. Wald,[3]
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, and University of Pittsburgh, Pittsburgh, Pennsylvania

MICE of the RF strain are unusually susceptible to the induction of myeloid leukemia by ionizing radiation (*1*). They have been used extensively in our laboratory, therefore, for studying the pathogenesis of myeloid leukemias, as compared with other leukemias and lymphomas. Our observations on the induction of leukemias and related growths by radiation, chemicals, and viruses are summarized in this report.

EFFECTS OF RADIATION

Age Distribution of Leukemias

Whole-body irradiation early in life advances the onset of thymic lymphomas, and to a lesser extent other neoplasms of reticuloendothelial origin in RF mice. The changes in age distribution are dependent on dose but do not affect all of these types of neoplasms to the same extent, the age-specific death rate from thymic lymphoma (text-fig. 1) and from myeloid leukemia (text-fig. 2) being affected more markedly than that from the other types of lymphomas and leukemias (text-fig. 3). Moreover, the age-mortality curves for thymic lymphoma and myeloid leukemia are not merely displaced but are altered in a more complex way; *i.e.*, both curves show two peaks, the first of which appears to be magnified more markedly by irradiation than the second. Whether this indicates

---

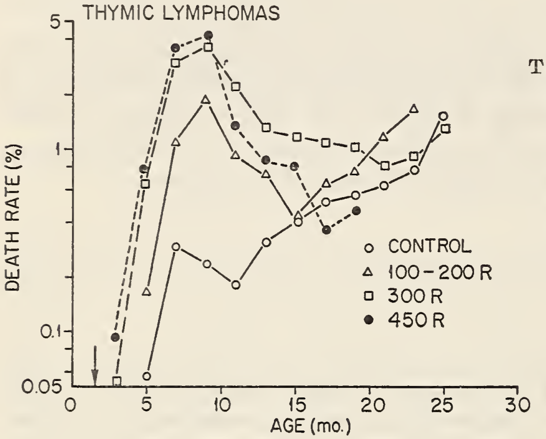
[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

[3] University of Pittsburgh.

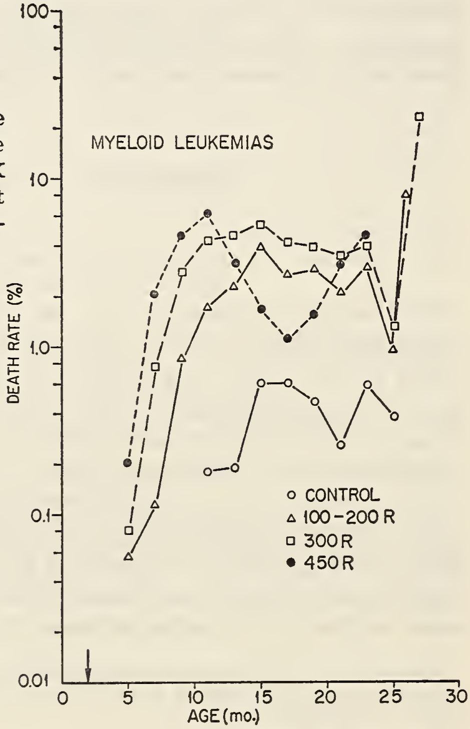
the existence of two or more different types of thymic lymphomas and myeloid leukemias, differing in etiology, or two or more subpopulations of mice differing in susceptibility to these growths remains to be determined.

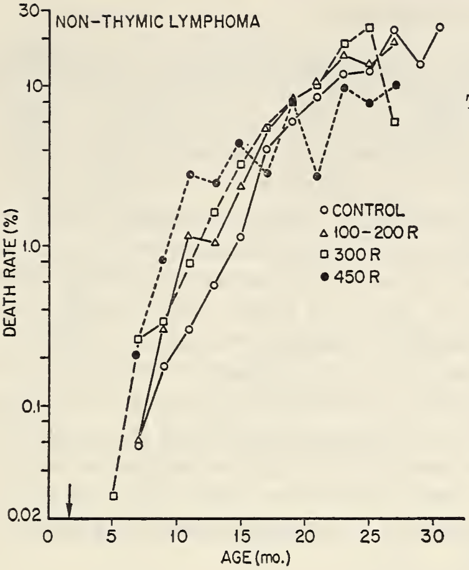
The effects of age at time of irradiation on the susceptibility of RF mice to the induction of leukemia are different for myeloid leukemia than for thymic lymphoma, as reported elsewhere (*3*). Because of evidence



TEXT-FIGURE 1.—Age-specific death rate from thymic lymphomas in RF male mice, as influenced by whole-body X irradiation. (*Arrow* denotes age at exposure.) From Upton, Kastenbaum, and Conklin (*2*).

TEXT-FIGURE 2.—Age-specific death rate from myeloid leukemia in RF male mice, as influenced by whole-body X irradiation. (*Arrow* denotes age at exposure.) From Upton, Kastenbaum, and Conklin (*2*).





Text-figure 3.—Age-specific death rate from nonthymic lymphomas in RF male mice, as influenced by whole-body X irradiation. (*Arrow* denotes age at exposure.) From Upton, Kastenbaum, and Conklin (*2*).

suggesting that the human embryo or fetus may be particularly sensitive to leukemia induction by irradiation (*4*), we have attempted to induce leukemia in RF mice by prenatal irradiation, with negative results thus far (table 1). As mentioned earlier (*5*), however, the comparative significance of these negative results is equivocal. If the association between prenatal irradiation and childhood leukemia incidence represents a cause-effect relationship, it is possible that smaller doses of radiation than those we used would have given positive results in our mice, *i.e.*, our smallest dose (50 r) was an order of magnitude higher than the dose presumed to be associated with leukemogenesis in the human embryo and fetus. On the other hand, the absence of a murine leukemia corresponding in age distribution to the acute leukemia of childhood may also explain our observations.

Table 1.—Incidence of leukemias in prenatally irradiated RF mice

X-ray exposure (r)	Gestational age at time of exposure (days)	Number of mice*	Mean age at death (days)	Incidence (%)		
				Myeloid leukemia	Thymic lymphoma	Other types of leukemia
0	9. 5	167	592	5	7	32
0	12. 5	229	607	3	8	36
0	17. 5	230	556	6	4	38
50	9. 5	231	589	2	5	38
50	12. 5	228	563	2	8	38
50	17. 5	227	562	1	5	46
150	9. 5	229	610	3	6	40
150	12. 5	224	622	3	6	39
150	17. 5	229	608	2	6	32

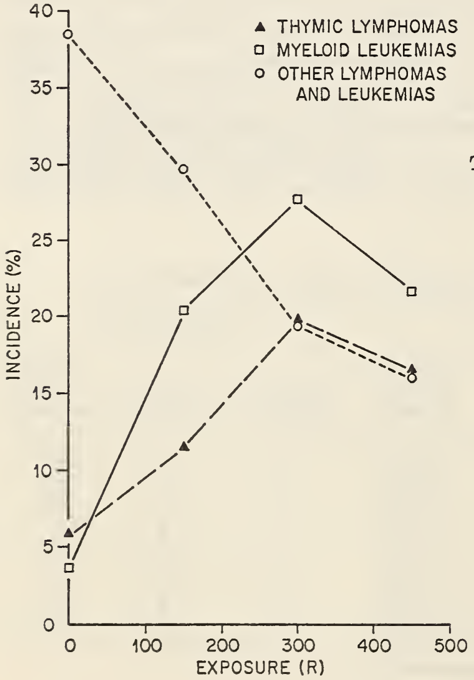
*Weanling males and females in approximately equal numbers.

Final Incidence

In association with their earlier onset, thymic lymphomas and myeloid leukemias are increased in final incidence by irradiation, but this is not true of the other types of lymphomas and leukemias (text-fig. 4). Why the incidence of the latter should be reduced by irradiation is unexplained, although it may conceivably result from depletion of the animal population at risk through mortality from earlier radiation-induced leukemias and other diseases. Efforts to affirm this interpretation, however, by statistical adjustment of the incidence to correct for intercurrent losses have failed (*6*). Nevertheless, many of the mice dying from thymic lymphoma or myeloid leukemia as a result of irradiation would have died from some other type of leukemia or lymphoma at a later age had they not been irradiated. To this extent, the effects of irradiation in many mice of the RF and certain other strains (*6*) may be considered merely to change the time of onset of leukemia and its hematologic type rather than to induce the disease in an animal that would otherwise have died without it. Viewed in this light, the relationship of the various types of leukemia to one another deserves further study.

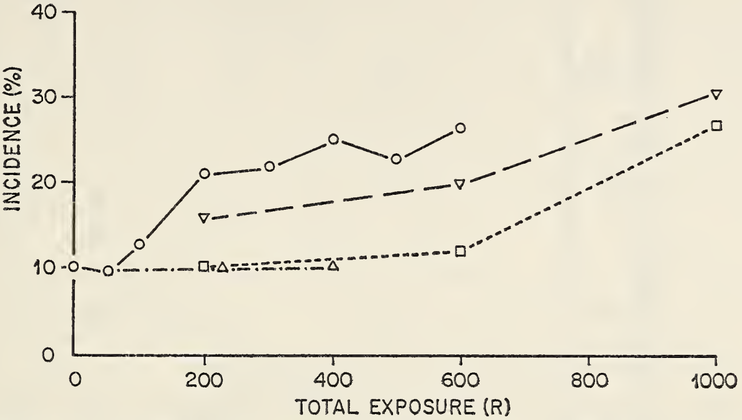
Dose-Incidence Relation

The relation between radiation dose and leukemia incidence is nonlinear over the range from 0 to 1000 rem. For both thymic lymphomas (text-fig.

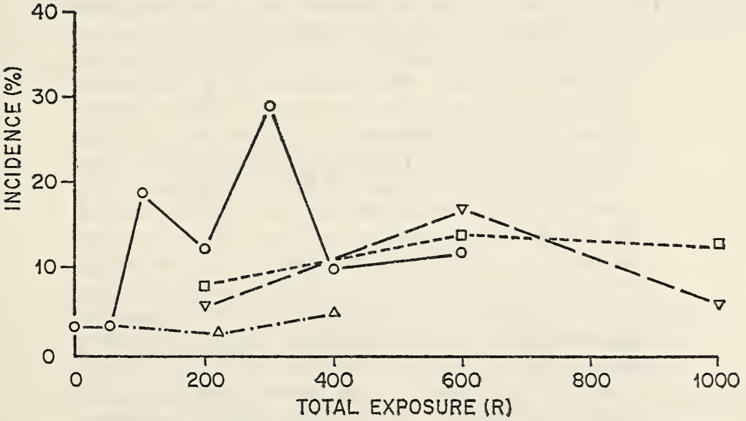


TEXT-FIGURE 4.—Incidence of various types of leukemias and lymphomas in RF male mice, in relation to X-ray dose. (Data are for the same mice as the age-incidence data plotted in text-figs. 1–3; each dose group contained 550–1900 animals.)

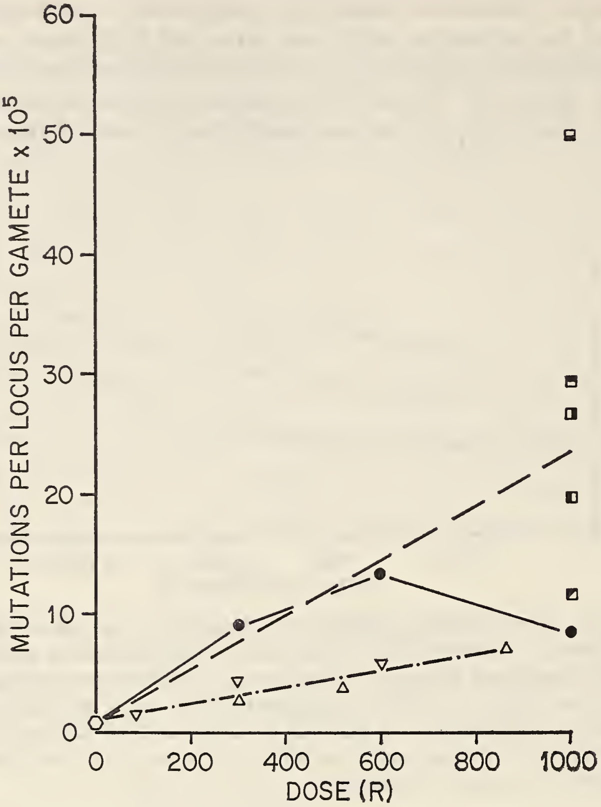
5) and myeloid leukemias (text-fig. 6), the dose-effect curves appear to be sigmoid and to saturate at high dose rates and high dose levels. With fractionation of the radiation exposure into several treatments, the saturation dose is increased. When the exposure is protracted over a long period at low dose rates, the leukemogenic effectiveness of the radiation is



Text-figure 5.—Dose-incidence relation for lymphomas in whole-body-irradiated female RF mice, as influenced by protraction or fractionation of irradiation. (All mice were 10 weeks old at start of irradiation.) ○ Single, acute exposure to Co^{60}-γ rays at 7 r per minute. △ Daily exposure to Co^{60}-γ rays at 0.005 r per minute. ▽ Two exposures to 300 kvp X rays, at 75 r per minute, 270 days elapsing between exposures. □ Ten exposures to 300 kvp X rays at 75 r per minute, 30 days elapsing between successive exposures.



Text-figure 6.—Dose-incidence relation for myeloid leukemia in whole-body-irradiated female RF mice, as influenced by protraction or fractionation of irradiation. (All mice were 10 weeks old at start of irradiation.) ○ Single, acute exposure to Co^{60}-γ rays at 7 r per minute. △Daily exposure to Co^{60}-γ rays at 0.0005 r per minute. ▽Two exposures to 300 kvp X rays, at 75 r per minute, 270 days elapsing between exposures. □Ten exposures to 300 kvp X rays at 75 r per minute, 30 days elapsing between successive exposures.



Text-figure 7.—Mutation rate in mouse spermatogonia, as influenced by dose and time-intensity factors of irradiation. From Russell (*8*). ⬡Nonirradiated control (531,500 offspring). ●X-irradiated, 90 r per minute (44,649–119,326 offspring per point). ▧X-irradiated, 90 r per minute, two exposures of 500 r each, separated by an interval of 2 hours (14,879 offspring). ◧X-irradiated, 90 r per minute, 5 exposures of 200 r each separated by an interval of 1 week (10,968 offspring). ◨X-irradiated, 90 r per minute, 5 exposures of 200 r each separated by an interval of 1 day (8,588 offspring). ⬒X-irradiated, 90 r per minute, 2 exposures of 600 r and 400 r separated by an interval of about 15 weeks (4,904 offspring). ⬓X-irradiated, 90 r per minute, 2 exposures of 500 r separated by an interval of 1 day (11,164 offspring). ▽γ-Irradiated, 0.001 r per minute (31,652–59,810 offspring per point). △γ-Irradiated, 0.009 r per minute, (24,281–58,457 offspring per point).

greatly reduced, at least in the case of γ rays, to the extent that leukemogenic effects may no longer be detectable. With fast neutrons, however, no such dose rate dependency has been noted; on the contrary, the leukemogenic effectiveness of these radiations appears to be relatively independent of dose rate (*7*).

Although the precise shapes of the dose-response curves cannot be inferred from our data, particularly at low dose levels, our results bear a strong resemblance to those for mutation induction in mouse spermatogonia (text-fig. 7). The true significance of the dose-effect curves remains to be determined, of course, but it is tempting to postulate that the saturation of the curves at high dose levels reflects excessive cytotoxicity to hematopoietic cells (*9*), possibly leading to selection of a resistant cell subpopulation, or excessive injury to other cells of the host, leading indirectly to inhibition of leukemia induction. This interpretation is consistent with the observed increase in saturation dose on fractionation of the exposure, since cell survival would be expected to increase with such fractionation through repair of sublethal radiation injury between successive doses (*10*). The reduced leukemogenic effectiveness of protracted, low-dose-rate γ-irradiation may be tentatively interpreted to mean that the leukemia-inducing effects of γ rays, like the mutagenic effects of these radiations, are reparable to a large degree if the radiations are absorbed gradually enough. Conversely, the dose-rate independence of the effects of fast neutrons implies that the neutron injury is relatively irreparable, as is envisioned for a variety of effects, *e.g.*, mutations in the mouse germ cells (*11*), killing of cells (*12*), and chromosome breakage (*13*).

Partial-body shielding has inhibited the induction of thymic lymphomas, and to a lesser extent myeloid leukemias, in RF mice (*14*); however, the quantitative aspects of the inhibition and the precise mechanism of the protection are ill defined.

EFFECTS OF CHEMICALS

Alkylating Agents

To determine whether the leukemogenic action of radiation can be mimicked by radiomimetic alkylating agents, several such chemicals have been administered to RF mice in large doses. Nitrogen mustard (HN_2), triethylenemelamine (TEM), Myleran, and 4-(*p*-dimethylaminostyryl)-quinoline (4M20) have been found to be leukemogenic to the thymus (table 2). None of these compounds has increased the incidence of myeloid leukemia under the conditions tested, however, although all have advanced the age distribution of the disease.

Because the doses of the compounds we used may have exceeded the optimum levels for induction of myeloid leukemia, we are investigating

TABLE 2.—Effects of alkylating agents on leukemia incidence in RF mice

Agent	Dose	Number of mice*	Thymic lymphoma		Myeloid leukemia		Other leukemias	
			Incidence (%)	Mean age at death (days)	Incidence (%)	Mean age at death (days)	Incidence (%)	Mean age at death (days)
Control †‡	—	2, 126	8	600	4	585	38	650
HN_2†	3.7–4.5 mg/kg	158	7	402	9	511	30	590
HN_2‡	2.4 mg/kg × 4	104	21	358	2	397	34	554
TEM†	3.0–4.0 mg/kg	146	16	360	6	557	31	563
TEM‡	1.5 mg/kg × 4	99	33	367	5	305	19	508
Myleran§	20.0 mg/kg	59	12	330	2	610	39	580
Myleran‡	12.0 mg/kg × 4	109	35	375	5	362	12	614
4M20‖	80–160 mg/kg	157	14	325	2	500	26	622

*Mice dying within 30 days after treatment were excluded. Mice were females, 10 weeks old at start of experiment.

†From Conklin *et al.* (*15*).

‡From Conklin *et al.* (*16*).

§From Upton *et al.* (*17*).

‖From Cosgrove *et al.* (*18*).

the effects of smaller doses of TEM, as well as the combined effects of TEM and X rays administered simultaneously. Until the results of these studies are available, it will not be possible to conclude that these chemicals differ from X rays in inducing only thymic lymphomas and not myeloid leukemias, particularly since myeloid leukemia has been induced in RF mice by other chemicals, *e.g.*, indol and related agents (*19*) and methylcholanthrene (*20*).

At present, although it is clear from these data that leukemogenic activity is shared by a variety of carcinogenic chemicals, as well as by radiation, the different agents appear to vary in relative leukemogenic potency for different types of hematopoietic cells, for reasons yet to be determined. The respective yields of thymic lymphomas and myeloid leukemias have been found to bear no discernible correlation with other toxic effects of the various agents, such as the LD50/30, degree of life shortening, carcinogenicity to nonhematopoietic organs, etc., but quantitative studies of the relative cytotoxic effects of the various agents on cells of the hematopoietic system have yet to be undertaken.

Effects of Urethan

In view of the observations that urethan may promote the induction of lymphomas by irradiation in adult mice (*21*, *22*) and may induce lymphomas when administered to newborn mice (*23*), we have explored the action of urethan on the development of myeloid as well as lymphoid leukemias in mice of the RF strain. Urethan administered alone or in combination with whole-body X radiation to young adult RF mice failed to alter the incidence of myeloid leukemia or thymic lymphomas (table 3). These results, which are consistent with the negative findings in cer-

Table 3.—Effects of urethan on leukemia incidence in irradiated and nonirradiated RF male mice

X-ray exposure (r)*	Urethan†	Number of mice	Median survival time (months)	Incidence (%) Thymic lymphoma	Myeloid leukemia	Other leukemia and lymphomas	Pulmonary adenoma
0	–	153	20	2	5	27	15
0	+	95	19	8	6	18	70
0	+‡	45	15	3	1	10	35
0	+§	47	16	2	8	18	92
100	–	39	14	0	7	8	9
100	+§	39	16	1	5	10	28
150	–	196	16	9	28	22	24
150	+	99	16	7	22	25	65
300	–	88	14	9	29	17	10
300	+	95	15	8	24	20	50

*Whole-body 300 kvp X irradiation (70-80 r/min; 0.44 mm Cu HVL) at 10 weeks of age.

†Urethan injected intraperitoneally, 1 mg per g, immediately before irradiation unless otherwise specified.

‡Urethan administered daily in the drinking water, 0.1 mg per ml.

§Urethan injected intraperitoneally, 1 mg per g, weekly for 5 weeks, beginning on the day after irradiation.

tain other strains of mice (*24*), suggest that the leukemogenic effects of urethan are strongly dependent on host factors or virus factors yet to be determined.

Effects of Amethopterin

As the first of a series of experiments to explore the effects of interference with nucleic acid metabolism on leukemia development, amethopterin was administered to RF mice in amounts approaching the acutely toxic level. The results from the small number of mice studied disclose no evidence of leukemogenic activity at the dose levels tested (table 4).

VIRAL ASPECTS

Viral Transmission: Effects of Estrogen

The cell-free transmission of radiation-induced myeloid leukemia (*25*, *26*), radiation-induced lymphoid leukemia (*27*), estrogen-induced lymphoid leukemia (*28*), and methylocholanthrene-induced lymphoid leukemia

Table 4.—Effects of amethopterin on leukemia development in RF mice

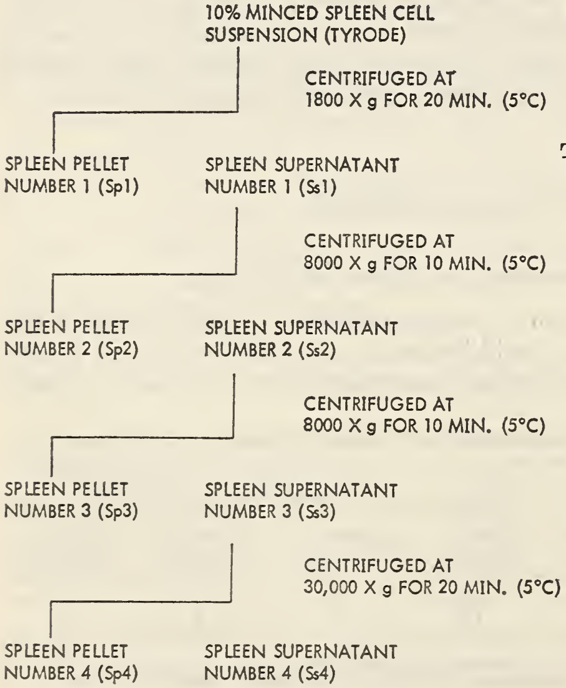
Treatment	Number of mice*	Mean survival time (months)	Leukemia incidence (%) Thymic lymphoma	Myeloid leukemia	Other leukemias
Controls	44	19	6	2	29
Amethopterin†	47	16	6	2	18

*The mice were RF males, 10 weeks old at the start of the experiment.

†Amethopterin (methotrexate) was administered daily in the drinking water, at a level of 0.02 mg per ml.

(*29*) in RF mice has indicated the involvement of a virus, or viruses, in the pathogenesis of these diseases. Moreover, the sex difference in the response of newborn RF mice to cell-free filtrates of myeloid leukemia tissue, *i.e.*, a higher incidence of myeloid leukemias induced in males and a higher incidence of thymic lymphomas induced in females, has suggested that sex factors influence the type of leukemia induced by the virus, or viruses, as well as the over-all incidence of the disease (*26*). These observations have been amplified in experiments with estrogen and testosterone (*30*), which show that estrogen inhibits the viral induction of myeloid leukemia in adult mice, whereas gonadectomy and testosterone have essentially no effect on it (table 5). This implies that the sex difference in the incidence of myeloid leukemia in virus-injected newborn (*26*) and irradiated adult (*31*) RF mice is attributable in large part to the difference in estrogen level. The mechanism by which estrogen lowers susceptibility to myeloid leukemia remains to be determined. Experiments to study the effects of the hormone on the dynamics of granulocytopoiesis and on immunity are planned.

Estrogen may also be implicated in the higher incidence of thymic lymphomas in virus-injected newborn (*26*) and irradiated adult (*31*) female RF mice, because of the leukemogenic action of estrogen on the thymus (*32*). The mechanism of the latter effect also remains, however, to be fully defined.


10% MINCED SPLEEN CELL SUSPENSION (TYRODE)
CENTRIFUGED AT 1800 X g FOR 20 MIN. (5°C)
SPLEEN PELLET NUMBER 1 (Sp1)
SPLEEN SUPERNATANT NUMBER 1 (Ss1)
CENTRIFUGED AT 8000 X g FOR 10 MIN. (5°C)
SPLEEN PELLET NUMBER 2 (Sp2)
SPLEEN SUPERNATANT NUMBER 2 (Ss2)
CENTRIFUGED AT 8000 X g FOR 10 MIN. (5°C)
SPLEEN PELLET NUMBER 3 (Sp3)
SPLEEN SUPERNATANT NUMBER 3 (Ss3)
CENTRIFUGED AT 30,000 X g FOR 20 MIN. (5°C)
SPLEEN PELLET NUMBER 4 (Sp4)
SPLEEN SUPERNATANT NUMBER 4 (Ss4)

TEXT-FIGURE 8.—Schematic diagram of procedure for preparing the ultracentrifugates from spleens of mice with myeloid leukemia. From Jenkins *et al.* (*30*).

TABLE 5.—Influence of sex, gonadectomy, and sex hormones on susceptibility to virus-induced myeloid leukemia in RF mice*

Ultracentrifugate	Hormone	Females						Males					
		Intact			Gonadectomized			Intact			Gonadectomized		
		Incidence†		Latency‡	Incidence		Latency	Incidence		Latency	Incidence		Latency
		(No.)	(%)	(days)	(No.)	(%)	(days)	(No.)	(%)	(days)	(No.)	(%)	(days)
None	None	0/1024§	—	—	0/118§	—	—	0/614§	—	—	0/103§	—	—
Ultracentrifugate	None	20/156	12. 8	64	—	—	—	19/188	16. 1	65	—	—	—
Ultracentrifugate	Vehicle	12/96	12. 5	67	14/96	14. 6	63	20/96	20. 8	64	20/96	20. 8	61
Ultracentrifugate	Testosterone	12/96	12. 5	66	14/96	14. 6	69	14/96	14. 6	56	18/96	18. 8	61
Ultracentrifugate	Estrogen	11/96	11. 5	70	5/96	5. 2	68	10/96	10. 4	66	15/96	15. 6	69

*Gonadectomy was performed at 3 weeks of age; ultracentrifugate of leukemic spleen [*i.e.*, Sp 3, Sp 4, and Ss 4 (*see* text-fig. 8)] was inoculated intravenously at 4 weeks of age; weekly intramuscular injections of 0.33 mg estradiol benzoate or 1 mg testosterone propionate were begun on the day of inoculation of ultracentrifugate.

†Number of mice developing myeloid leukemia within 90 days, over the number injected.

‡Mean number of days after ultracentrifugate injection until development of the disease.

§Data from Upton *et al.* (*31*) and Jenkins and Upton (*26*).

Donor Variation in Virus

We have observed considerable variability among leukemic donors in the leukemogenic potency of the cell-free material extractable from their tissues, as exemplified by the data summarized in table 6. These data show that passage materials obtained from donors rendered leukemic by injection of 30,000 × *g* spleen centrifugates (Ss 4) are less potent than those obtained from donors rendered leukemic by injection of 30,000 × *g* spleen pellets (Sp 4). These results disclose that the type of inoculum inducing leukemia in a given mouse may determine the amount and/or potency of the virus subsequently recoverable from the animal's tissues. This effect may conceivably depend either on the amount of virus injected or on other factors, such as the possible presence of helper or inhibitor viruses. The data available at present do not exclude any of these possibilities.

TABLE 6.—Leukemogenic potency of donor spleen ultracentrifugates in relation to type of passage material responsible for leukemia in donor*

Passage material that caused leukemia in donor	Fraction and percentage of recipients developing leukemia†					
	Sp 3 (2d 8,000 × *g* pellet)		Sp 4 (30,000 × *g* pellet)		Ss 4* (30,000 × *g* centrifugate)	
	(No.)	(%)	(No.)	(%)	(No.)	(%)
Sp 3	1/8	12	74/200	37	6/40	15
Sp 4	5/72	7	80/326	24	44/213	21
Ss 4	1/75	1	55/314	18	1/235	0.4

*For definition of terms and schematic description of procedure for preparing passage materials, *see* text-figure 8.

†The results tabulated are based on the combined data from several dozen separate passages. The mice used were young adult RF males and females given injections and scored according to the techniques described elsewhere (*30*).

Storage of Virus

Because of the variable potency of our passage materials, as mentioned in the foregoing paragraph, our efforts to study the cell-free transmission of radiation-induced myeloid leukemia in RF mice have been hampered by our inability to preserve the activity of passage materials in a frozen state and thereby to stockpile and pool materials of known potency. Our attempts to freeze the virus by methods that can be satisfactorily applied to the Gross (*33*) and Moloney (*34*) viruses have been unsuccessful. Recently, however, we have succeeded in preserving the leukemic cells by slow freezing in a 10 percent solution of dimethylsulfoxide (table 7). Such cells have remained viable at liquid nitrogen temperature and yielded active virus on thawing (table 7). With this improved technique, we are now stockpiling leukemogenic passage materials in the frozen state and striving to select for increasingly potent lines of virus on serial passage.

TABLE 7.—Effects of various freezing methods on the leukemogenic potency of passage materials

Method of freezing		Type and potency of frozen-thawed passage material				
Suspending medium	Rate of chilling	Sp 1	Sp 3	Sp 4	Ss 4	Plasma
		(fraction of recipients developing myeloid leukemia)				
Saline or Tyrode's	Rapid*		0/36	1/95		0/35
Tyrode's	Slow				0/59	
Potassium citrate	Rapid†				0/78	0/92
Glycerol	Slow	1/35				
Dimethylsulfoxide	Slow‡	21/129	26/136§	17/140§	6/90§	

*Details of the method are described by Gross (*33*).

†Details of the method are described by Moloney (*34*).

‡Details of the method are described by Albright *et al.* (*35*).

§Ultracentrifugate itself not frozen but prepared from cell suspensions frozen in dimethylsulfoxide and subsequently thawed.

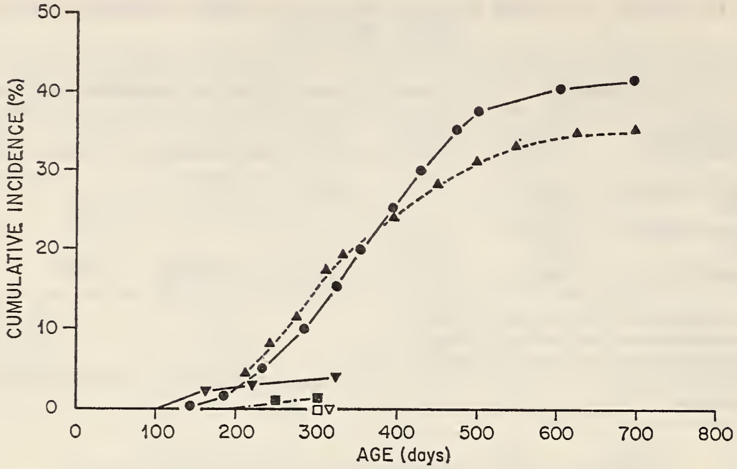
Response of RF Mice to Other Leukemia Viruses

In contrast to our radiation myeloid leukemia virus (RMLV), the Rauscher virus (kindly supplied by Dr. F. J. Rauscher of the National Cancer Institute) has failed to produce myeloid leukemias or thymic lymphomas in RF recipients. Instead, in all of the 65 conventional and 21 germfree RF recipients examined thus far, it has produced reticulum cell sarcomas, with erythroblastosis. Similarly, the Gross Passage A virus (kindly supplied by Dr. Ludwik Gross of the Veterans Administration Hospital, Bronx, N.Y.) has produced only thymic lymphomas in the limited number of RF recipients we have injected to date, although it has been reported to increase the incidence of myeloid leukemia in irradiated RF/J mice (*36*).

Our results, therefore, clearly set the RMLV apart from the Rauscher and Gross viruses in the character of the leukemias induced in nonirradiated RF recipients. Other preliminary results disclose differences in antigenic specificity between the leukemias induced in RF mice by the RMLV and those induced by the Gross and Rauscher viruses (L. J. Old and E. A. Boyse, personal communication).

INFLUENCE OF MICROFLORA

Although a high incidence of thymic lymphomas may be induced in germfree RFM/Unf mice by 300 r whole-body X irradiation (*37*), not a single case of myeloid leukemia has been noted in 82 such males and 98 such females observed for more than 14 months after exposure. By contrast, at least 20 cases would have been expected within the same period in a comparable number of conventionally reared RF males (text-fig. 9). In specific-pathogen-free (SPF) RF mice, the incidence of myeloid leukemia appears to be similarly reduced but the rate of induction of thymic



TEXT-FIGURE 9.—Cumulative incidence of myeloid leukemia in 300 r-irradiated germfree, SPF, and conventional RF male mice in relation to age. ●Conventional noninbred RF/Un mice (1,905 mice, irradiated at 5–10 weeks of age, 1956–63). ▲Conventional inbred RFM/Un mice (46 mice, irradiated at 5–8 weeks of age, 1956–62). ▼Conventional inbred RFM/Un mice (99 mice, irradiated at 5–6 weeks of age, 1964–65). ■Conventional inbred RFM/Un mice (70 mice, irradiated at 10 weeks of age, 1964–65). ▽Germfree inbred RFM/Unf mice (82 mice, irradiated at 5–6 weeks of age); from Walburg *et al.* (*37*). □SPF, inbred RFM/Unf mice (38 mice, irradiated at 10 weeks of age, 1964–65) (L. J. Serrano, R. C. Allen, T. T. Odell, Jr., and G. E. Cosgrove, Jr., private communication).

lymphomas comparably high. From these preliminary results, our germfree and SPF mice appear to be less susceptible than their conventionally reared counterparts to induction of myeloid leukemia but not of thymic lymphoma.

The possibility that this difference results from depressed granulocytopoietic activity is being explored, since the leukocyte count in our germfree and SPF mice is significantly lower than in their conventionally reared counterparts (H. E. Walburg, Jr., and T. T. Odell, Jr., unpublished data).

Other conceivable explanations for the reduced incidence of myeloid leukemia in our germfree and SPF mice include viral and genetic changes. For example, our germfree mice, and the SPF mice derived from them, may possibly lack a virus essential for the development of myeloid leukemia. Repeated serological testing of the germfree RF colony has shown no evidence of the conventional mouse viruses. Leukemia-virus-like particles have, however, been found in the tissues of our germfree mice with lymphomas, on electron microscopic examination (*37*). These particles may be the agent responsible for the lymphomas, but this has not yet been proven by bioassay. If cell-free extracts are found to transmit the lymphomas in germfree mice, it will be of interest to determine whether they can induce myeloid leukemias as well as lymphomas in conventional RF mice. In the meantime, our germfree RF mice are being injected with cell-free myeloid leukemia extracts to determine whether they will develop myeloid leukemia under these conditions as their conventional counterparts do.

There is also the possibility that genetic changes are responsible for the reduced incidence of myeloid leukemia in our germfree mice. In establishing our germfree line, which was accomplished by cesarian section of a single RF litter (from which our SPF line has since been derived), we may inadvertently have selected for a genetically less susceptible subline. We may have made a similar type of selection, for that matter, before the establishment of our germfree subline, through our practice of extending our foundation core from a single breeding pair every fifth generation. Consistent with this possibility is the seemingly lower incidence of myeloid leukemia in our conventionally reared RF mice today than that to be expected from our experience with their predecessors (text-fig. 9). Even our conventional mice, however, have presumably undergone substantial changes in microflora within recent years because of profound improvements in animal husbandry and housekeeping in our animal colony. Hence, we cannot weigh the relative influence of genetic, as opposed to microfloral, changes in the observed differences in leukemia incidence without further study.

SUMMARY

Mice of the RF strain treated with ionizing radiation, nitrogen mustard, triethylenemelamine, or Myleran showed a markedly increased incidence of myeloid leukemia, or thymic lymphoma, or both, depending on their age, sex, microflora, and physiological state when treated, as well as on the conditions of treatment. Urethan, with or without irradiation, has not affected the incidence of either disease. Myeloid leukemias induced by irradiation have been serially transmitted in adult, as well as newborn, recipients by cell-free extracts. Such extracts also increase the incidence of thymic lymphomas. Reticulum cell sarcomas alone have been induced by the Rauscher virus. In general, females are less susceptible to the induction of myeloid leukemia than males, which may be attributable at least in part to the effects of estrogen since this hormone inhibits viral transmission of the disease. Germfree mice appear less susceptible to the induction of myeloid leukemia by irradiation than their conventionally reared counterparts, but are no less susceptible to induction of thymic lymphomas. These observations indicate that a complex interaction among host factors and extraneous agents determines the incidence, latency, hematologic type, clinicopathologic features, and transmissibility of murine leukemia in mice of the RF strain.

REFERENCES

(*1*) Upton, A. C., and Furth, J.: Host factors in the pathogenesis of leukemia in animals and in man. *In* Proceedings of the Third National Cancer Conference, The American Cancer Society, Inc. Philadelphia, J. B. Lippincott Company, 1957, pp 312–324.

(*2*) Upton, A. C., Kastenbaum, M. A., and Conklin, J. W.: Age-specific death rates of mice exposed to ionizing radiation and radiomimetic agents. *In* Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation (Harris, R. J. C., ed.). London, Academic Press Inc, 1963, pp 285–297.

(*3*) Upton, A. C., Odell, T. T., Jr., and Sniffen, E. P.: Influence of age at time of irradiation on induction of leukemia and ovarian tumors in RF mice. Proc Soc Exp Biol Med 104: 769–772, 1960.

(*4*) MacMahon, B.: Statement in Hearings on Fallout, Radiation Standards and Countermeasures, Part II, pp 594–601. Congress of the United States, 88th Congress, 1st Session, August 20–22 and 27, 1963.

(*5*) Upton, A. C.: Comparative aspects of carcinogenesis by ionizing radiation. Nat Cancer Inst Monogr 14: 221–242, 1964.

(*6*) Upton, A. C., Kimball, A. W., Furth, J., Christenberry, K. W., and Benedict, W. H.: Some delayed effects of atom-bomb radiation in mice. Cancer Res 20: 1–62, 1960.

(*7*) Upton, A. C., Randolph, M. L., Darden, E. B., Jr., and Conklin, J. W.: Relative biological effectiveness of fast neutrons for late somatic effects in mice. *In* Biological Effects of Neutron and Proton Irradiations, vol 2. Vienna, International Atomic Energy Agency, 1964, pp 337–345.

(*8*) Russell, W. L.: The effect of radiation dose rate and fractionation on mutation in mice. *In* Repair from Genetic Radiation (Sobels, F. H., ed.). Oxford, Pergamon Press, 1963, pp 205–217.

(*9*) Reif, A. E.: Radiation carcinogenesis at high dose-response levels. A hypothesis. Nature (London) 190: 415–417, 1961.

(*10*) Elkind, M. M., and Sinclair, W. K.: Recovery in X-irradiated mammalian cells. *In* Current Topics in Radiation Research, vol 1 (Ebert, M., and Howard, A., eds.). Amsterdam, North-Holland Publishing Company, 1965, pp 168–220.

(*11*) Russell, W. L.: Studies in mammalian radiation genetics. Nucleonics 23: 1965.

(*12*) Fowler, J. F., and Morgan, R. L.: Pre-therapeutic experiments with the fast neutron beam from the Medical Research Council cyclotron. VIII. General review. Brit J Radiol 36: 115–121, 1963.

(*13*) Lea, D. E.: Actions of Radiations on Living Cells. Cambridge, University Press, 1947.

(*14*) Odell, T. T., Jr., Cosgrove, G. E., and Upton, A. C.: Modification of delayed somatic effects of ionizing radiation. *In* Radiation Protection and Recovery (Hollaender, A., ed.). Oxford, Pergamon Press, 1960, pp 303–315.

(*15*) Conklin, J. W., Upton, A. C., Christenberry, K. W., and McDonald, T. P.: Comparative late somatic effects of some radiomimetic agents and X-rays. Radiat Res 19: 156–168, 1963.

(*16*) Conklin, J. W., Upton, A. C., and Christenberry, K. W.: Further observations on late somatic effects of radiomimetic chemicals and X-rays in mice. Cancer Res 25: 20–28, 1965.

(*17*) Upton, A. C., Wolff, F. F., and Sniffen, E. P.: Leukemogenic effect of Myleran on the mouse thymus. Proc Soc Exp Biol Med 108: 464–467, 1961.

(*18*) Cosgrove, G. E., Davis, M. L., and Asano, M.: Oncogenic effects of 4-(*p*-dimenthylaminostyryl)quinoline (4M20) in mice. Cancer Res 25: 938–945, 1965.

(*19*) Ehrhart, H. V., Stich, W., and Benoit, W.: Untersuchungen über experimentelle Leukämien. IV. Mitteilung: Steigerung der Leukämiehaufigkeit bei RFH-Mäusen nach Erhöhung der Indoldosis. Blut 6: 11–17, 1960.

(*20*) Irino, S., Sezaki, T., and KO, I.: Induction in RF mice of myelogenous leukemia by oral administration of 20-methylcholanthrene and the co-leukemogenic action of croton oil. Igaku To Seibutsugaku (Med Biol, Tokyo) 68: 206–212, 1964.

(*21*) Berenblum, I.: Radiation, urethane, and a 'transmissible factor' in experimental leukaemogenesis. Acta Un Int Cancr 20: 893–898, 1964.

(*22*) Lieberman, M., Haran-Ghera, N., and Kaplan, H. S.: Potentiation of virus leukaemogenesis in C57BL mice by X-irradiation of urethane. Nature (London) 203: 420–422, 1964.

(*23*) Toth, B., Della Porta, G., and Shubik, P.: The occurrence of malignant lymphomas in urethane-treated Swiss mice. Brit J Cancer 15: 322–326, 1961.

(*24*) Law, L. W., and Precebutti, A.: Inhibition by urethane (ethyl carbamate) of virus induction of leukemia in C3H mice. Nature (London) 200: 692–693, 1963.

(*25*) Parsons, D. F., Upton, A. C., Bender, M. A., Jenkins, V. K., Nelson, E. S., and Johnson, R. R.: Electron microscopic observations on primary and serially passaged radiation-induced myeloid leukemias of the RF mouse. Cancer Res 22: 728–730, 1962.

(*26*) Jenkins, V. K., and Upton, A. C.: Cell-free transmission of radiogenic myeloid leukemia in the mouse. Cancer Res 23: 1748–1755, 1963.

(*27*) Hiraki, K., Irino, S., Sota, S., and Ikejiri, K.: Leukemogenic activity of cell-free filtrates from radiation-induced leukemia of RF mice. Acta Haemat Jap 25: 816–821, 1962.

(*28*) Kunii, A., Takemoto, H., and Furth, J.: Leukemogenic filterable agent from estrogen-induced thymic lymphoma in RF mice. Proc Soc Exp Biol Med 119: 1211–1215, 1965.

(*29*) Hiraki, K., Irino, S., Sezaki, T., and Suzaki, M.: Success in cell-free transmission of 20-methylcholanthrene-induced RF mouse leukemia (in Japanese). Acta Haemat Jap 25: 822–830, 1962.

(*30*) Jenkins, V. K., Odell, T. T., Jr., and Upton, A. C.: Effect of sex hormones on susceptibility to viral transmission of myeloid leukemia in the mouse. Cancer Res 26: 454–458, 1966.

(*31*) Upton, A. C., Wolff, F. F., Furth, J., and Kimball, A. W.: Comparison of the induction of myeloid and lymphoid leukemias in X-radiated RF mice. Cancer Res 18: 842–848, 1958.

(*32*) Kaplan, H. W., Nagareda, C. S., and Brown, M. B.: Endocrine factors and radiation-induced lymphoid tumors in mice. *In* Recent Progress in Hormone Research 10 (Pincus, G., ed.). New York, Academic Press Inc., 1954, pp 293–338.

(*33*) Gross, L.: Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proc Soc Exp Biol Med 94: 767–771, 1957.

(*34*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*35*) Albright, J. F., Makinodan, T., and Mazur, P.: Preservation of antibody-producing cells at low temperatures: A method of storage that allows complete recovery of activity. Proc Soc Exp Biol Med 114: 489–493, 1963.

(*36*) Yokoro, K., Takemoto, H., and Kunii, A.: Role of mouse lymphoma virus in induction of myeloid leukemia by X-ray. Ann NY Acad Sci 114: 203–212, 1964.

(*37*) Walburg, H. E., Jr., Upton, A. C., Tyndall, R. L., Harris, W. W., and Cosgrove, G. E.: Preliminary observations on spontaneous and radiation-induced leukemia in germfree mice. Proc Soc Exp Biol Med 118: 11–14, 1965.

DISCUSSION

Dr. Siegler: Dr. Upton stated that there is no counterpart of acute lymphatic leukemia of childhood in mice. I think it is difficult to draw that conclusion yet because we know so little about the pathogenesis of leukemia in man. If the comparison is made on the basis of time of onset, then virus-inoculated mice develop leukemia at age 60 days, which is equivalent to 5 to 7 years of age in humans. If the comparison is made on the basis of pathogenesis, then this is a difficult problem because what one sees clinically in children represents only the end stage of the disease. While we know very well the *pathology* of human leukemia, we know almost nothing of its *pathogenesis.* We know much more about mouse pathogenesis and possibly ultimately can say more definitely whether there is such a counterpart in man.

Dr. Upton: I would only say that I do not think the matter is closed in mice of the RF strain or other strains we have worked with. We haven't seen acute leukemias during the period of infancy or adolescence in the mouse. The earliest forms of leukemia we've seen arise as thymic lymphosarcomas and may or may not generalize to involve the blood stream before the death of the animal. Clearly, one of the fundamental questions is the relationship among the different hematologic types of leukemia which we encounter in the mouse. Are they variants of one disease with common mechanisms of etiology? To what extent do they differ in their pathogenesis? Until we can answer these questions, it is a very difficult matter to extrapolate from mouse to man.

Dr. Stansly: If Dr. Upton wants to study myeloid leukemia in mice, I suggest he try our virus in BALB/c mice because we get myeloid leukemias almost exclusively.

An additional point I'd like to make is that we have observed a very striking sex effect in our myeloid leukemias, but it is in the opposite direction from Dr. Upton's observation in that we see about twice as high an incidence in females as in males in the BALB/c and MABA strains.

Dr. Salaman: Dr. Upton made the interesting observation that the specific-pathogen-free (SPF) and germfree mice got no myeloid leukemia after irradiation and he suggested this might have been because he had selected a resistant strain of mouse at the same time as he developed his SPF strain. Dr. Upton, could you not test this possibility by selective addition of intestinal flora, perhaps even to give individual organisms and see which organism is responsible for the difference?

Dr. Upton: I thank Dr. Stansly for his remarks and hope I can get together with him later.

In response to Dr. Salaman's question, we are now in the process of injecting germfree recipients with ultracentrifugates that produce myeloid leukemia in conventional animals, to see whether putting in the virus, or presumably doing so, would in fact produce the disease we get in the conventional animal. The results are not yet available.

The cesarian-derived subline has been allowed to become contaminated, and we are going to irradiate some of these contaminated animals to see whether the differences we've observed are in fact the result of genetic selection in deriving the subline or whether they can be ascribed to floral differences. Of course, they may derive in part from both differences.

Dr. Block: I have questions for Dr. Upton. In consideration of the effect of radiation on embryos I notice that in your slide you used a much lower dose, 50 r, for animals 9 to 10 days of age of intrauterine gestation as opposed to 150 r. This might affect the results just by virtue of the dosage.

The other thing is that you ought to consider whether the irradiation effect on embryos is not just a manifestation or relationship with the age of the embryo. There are certain critical periods in embryonic life when the animal may be particularly susceptible to induction of radiation—when the thymus is first forming—as opposed to older animals in which the thymus has already formed, or perhaps the time when the bone marrow first forms might again be a very critically important period. Have you considered these phenomena: in other words, irradiation not only in terms of age, but in terms of particular stages of development in the embryo at the time of radiation?

Dr. Upton: We did in fact strive to irradiate at three postconception ages that would correspond to the yolk sac stage of the hematopoietic system, the intrahepatic stage, and, finally, the beginning of the marrow stage. The times, as selected by our coinvestigator in this work, Dr. R. A. Popp, were 9½, 12½, and 17½ days. We chose 150 and 50 r because earlier experiments at higher levels had been negative and we wanted to work down to lower doses.

This doesn't mean, of course, that dropping the dose another factor of 10 to 5 rads would produce the same negative results, but so far at least we've seen nothing.

I'm not sure when the thymus develops in the mouse. I think your point is well taken. It would be worthwhile to look into that.

Occurrence of Low Leukemogenic but Immunizing Mouse Leukemia Virus in Tissue Culture[1, 2]

Joseph G. Sinkovics, Barbara A. Bertin, *and* Clifton D. Howe,[3] *Section of Clinical Tumor Virology and Immunology, Department of Medicine, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas*

In August 1964, the Rauscher mouse leukemia virus, in the form of a cell-free extract of leukemic mouse spleen, was inoculated into a tissue culture of normal Swiss suckling mouse thymus and spleen cells in our laboratory. During the past 14 months, this cell line has undergone more than 42 passages. Several interesting phenomena relevant to the relationship of a leukemogenic virus and host cells *in vitro* emerged and are being investigated. It has already been briefly reported that the leukemogenic activity of tissue culture fluids from advanced passages may be minimal, despite the fact that replicating leukemia virus is present in the cultures (*1*). Recent publications on a very similar observation (*2*,*3*) prompted this present report.

MATERIALS AND METHODS

Tissue cultures.—The mixed spleen and thymus culture from healthy suckling Swiss mice was prepared without trypsinization. A litter of 4-day-old mice was killed. Their spleens and thymuses were aseptically removed, very finely minced, and explanted on the glass in small T flasks and Leighton tubes.

Mouse embryo tissue cultures were prepared with cells trypsinized off from torsos of 2½-week fetuses of Swiss mice. Hanks' balanced salt solution with vitamins and amino acids (Eagle's medium) was supple-

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This work was supported by Public Health Service grant CA-7923 from the National Cancer Institute and award 5-K3-CA-16; 747 to the senior author, and by institutional grant FR-05511-In-17 from the American Cancer Society, Inc.

[3] We are obliged to Mrs. Doris Gaines and to Mr. David Grant for assistance in these studies.

mented by 0.5% lactalbumin hydrolysate and 20% inactivated calf serum. The medium contained 200 units of penicillin, 0.2 mg streptomycin, and 0.1 mg neomycin per ml. No microorganisms could be isolated from the cultures described herein on blood agar plates and on Difco (Difco Laboratories, Detroit, Mich.) PPLO plates.

Cells were transferred by trypsinization. The procedure recommended by Merchant *et al.* (*4*) was carried out. From the 35th passage on, the cultures were treated also with Versene before trypsinization. Approximately 10^4 cells or more were used to initiate new cultures.

Interferon assay.—No elaborate efforts (acidification and realkalinization, dialysis, etc.) were made to identify interferon. In order to determine the presence of an inhibitory substance, supernatants of centrifuged (3000 rpm for 20 minutes) tissue culture fluids were used for pretreatment of healthy mouse embryo monolayers. Monolayers consisting of about 10^6 cells were overlayed with 0.5 ml of such supernatants and incubated at 37° C for 6 and 30 hours, respectively. Thereafter, the cultures were rinsed with tissue culture medium containing 2% inactivated calf serum. After withdrawal of fluid, the cultures were incubated with predetermined doses (fifty 50% cytopathic units in 0.1 ml volume) of vesicular stomatitis pseudorabies (Aujeszky) viruses. Incubation at 37° C for 1 hour followed; then free virus was rinsed off, and nutrient medium was replaced. The cultures were incubated at 37° C for 48 hours, at which time the cytopathic effect was recorded and tissue culture fluids were harvested and stored rapidly by being deep frozen in dry ice. In a separate experiment, serial dilutions ranging from undiluted to dilution 10^{-7} of the above fluids were inoculated into new mouse embryo monolayers, with 4 to 5 cultures per dilution. These cultures were incubated at 37° C for 4 days and the highest virus dilution still able to cause cytopathic effect was determined. Certain cultures inoculated with high dilutions often suffered not more than 25% cytopathic effect of their surface area; they were counted as positive. The highest virus dilution causing cytopathic effect in 50% of the cultures inoculated was determined by the Reed-Muench method (*5*). These titers were then compared, and differences between virus yield from cultures pretreated with various experimental and control tissue culture fluids were thus calculated.

Heat-inactivated (56° C for 30 minutes) sera of adult Swiss mice were used for positive interferon control preparation, as recommended by Baron and Buckler (*6*). These mice were inoculated intravenously with the suckling mouse brain adapted RO strain of the Newcastle disease virus (*7*) and were bled 6 hours later.

Direct interference studies were carried out as follows: Approximately equal numbers of cells from the cell culture line, harboring the Rauscher virus (line #3), and from the cell culture, parallelly maintained from the same source of cells but never intentionally infected with Rauscher virus (line #4), were cultured in Leighton tubes. These cultures were inoculated with equal doses of vesicular stomatitis and pseudorabies

(Aujeszky) viruses, respectively. After 1-hour incubation, the free viruses were rinsed off and the cultures were incubated for 24 to 36 hours. The cytopathic effect was recorded, and the culture fluids were harvested and titrated for vesicular stomatitis or pseudorabies virus content in new mouse embryo monolayers.

Mouse inoculations.—For bioassays, *i.e.*, detection of leukemogenic activity of fluids from serially passaged cell lines, 3-week-old Swiss mice, from the Texas Inbred Mouse Company (Timco), Houston, Texas, were used. The incidence of spontaneous leukemia in these mice is far less than 0.5% at 1 year of age. Inoculations were given intraperitoneally. All mice were ear numbered and observed for 7 to 10 months. All mice were autopsied, and print preparations or histologic sections were made to confirm diagnosis of leukemia.

Cytotoxic tests.—Heat-inactivated (56° C for 20 minutes) mouse sera or their serial dilutions, prepared with saline in a volume of 0.05 ml, were mixed with approximately $10^{5.7}$ fresh, suspended Rauscher leukemic spleen cells in a volume of 0.05 ml, and 0.05 ml fresh guinea pig serum diluted 1:5 was added. The mixture was incubated in the presence of 0.05 ml, 1:1,000 dilution, eosin Y at 37° C for 45 to 60 minutes. A sample of the cell suspension was transferred into hemocytometer, and the number of swollen pink cells per 100 cells was counted.

Viruses.—The Rauscher virus was received in 1962 from Dr. F. J. Rauscher in the form of a deep-frozen leukemic mouse spleen extract. It is being passaged in Timco Swiss mice in our laboratory. Cell-free extracts of weighed, ground leukemic spleens were prepared with chilled tissue culture medium containing 2% inactivated calf serum. The extracts were centrifuged in the cold at 1500 rpm for 15 minutes; the supernatant was centrifuged again at 3000 rpm for 20 minutes. The second supernatant was distributed in small vials and stored by being rapidly deep frozen in dry ice.

The vesicular stomatitis virus was received from Dr. W. Henle in the form of infected chicken embryo tissue culture fluid. The virus is being passed in mouse embryo cells *in vitro* in our laboratory.

The pseudorabies (Aujeszky) virus was given to us by Dr. W. Munyon; the strain derives from Dr. A. Kaplan and is being maintained here in mouse embryo cells *in vitro*.

RESULTS

Tissue Culture Lines

Since our previous report (*8*), three newer attempts were made to maintain the Rauscher virus through serial passages in Swiss mouse cells grown *in vitro*.

In the first and second attempts, fresh Swiss mouse embryo monolayers were inoculated with a cell-free spleen extract from leukemic Swiss mice.

Medium was changed at 5-day intervals. The *fluid* was transferred to new mouse embryo monolayers at 2- to 3-week intervals. These culture lines (#1 and #2) were discontinued after the tenth passage. The fluids used for passage were also bioassayed for leukemogenic activity in mice and later used for immunization experiments. Uninoculated fluids from mouse embryo cultures were also passed and used as controls.

The third tissue culture line (#3) was derived from aseptically removed spleens and thymuses of Timco Swiss suckling mice. The Rauscher virus used for inoculation of this culture line was derived from a 20% cell-free spleen extract of fully leukemic mice and was stored as stock virus in small vials on dry ice. The LD50 of this extract was approximately a 10^{-4} dilution, if 0.1 ml intraperitoneal doses were used for titration in 3-week-old Timco Swiss mice. The virus in 0.2 ml volume per small T flask (approximately $10^{3.7}$ mouse LD50) was left on the cell layer for 1 hour, while the cultures were incubated at 37° C. The number of cells in such a culture was approximately 10^5. The leukemic spleen extract was then rinsed off and nutrient medium was replaced. The cultures were incubated at 37° C; the medium was changed at 5- to 7-day intervals; cultures were trypsinized and the *cells* were transferred to initiate new cultures every 10 to 14 days. After the third passage, the culture consisted of fibroblastic cells and of a smaller, less elongated cell type, probably reticulum cell. Between cell passages 12 and 35, multilayers and cell growth in a very irregular fashion (fig. 1) were often observed. In later passages, the tendency to form monolayers became better established (fig. 2). Pyknosis and death of individual cells occurred in crowded, old cultures; young cultures grew vigorously without cytopathic effect. It has been observed that the leukemogenic effect of tissue culture fluids in mice ceased or became very erratic after the seventh passage of the cells; especially the incubation period of leukemia lengthened. At the same time, initiation of new passages with trypsinized cells of the culture became increasingly difficult. The transferred cells spread out well in the new culture flasks but failed to divide. Many cells (perhaps more than 75%) lost viability during transfer and far more than 10^4 cells were needed to initiate a new culture. An attempt was made to refresh the cultures by addition of fresh Timco Swiss mouse embryo cells. The addition of about equal numbers of healthy mouse embryo cells, obtained from minced torsos by trypsinization, to cells from advanced passages of the original culture line restored the healthy appearance of the cultures and the leukemogenic activity of fluids harvested from such joined cultures. This procedure will be referred to as "rejuvenation."

Only half of the originally prepared cultures of suckling Swiss mouse thymus and spleen were inoculated with the Rauscher virus. Control cultures (not inoculated with Rauscher virus but overlayed for 1 hour with normal mouse spleen extract that was then rinsed off and replaced with nutrient medium), referred to here as line #4, has undergone 35 cell passages at this writing. The culture consists of small, slightly elongated cells, probably reticulum cells and fibroblasts, growing in monolayers (fig. 3). Early cell passages (*i.e.*, passages 13 and 19) of the control culture line were also "rejuvenated" with the same normal mouse embryo cells used for line #3.

Bioassays

During passages, fluids were collected from the above-described culture lines; most fluids tested in bioassay were harvested 10 to 15 days after the initiation of the culture, *i.e.*, when the cells formed a sheet. These fluids were rapidly deep frozen on dry ice and acetone and stored in a dry-ice chest. Bioassays were periodically carried out in 3-week-old Timco Swiss mice by the intraperitoneal inoculation of 0.5 ml of rapidly thawed fluids. Table 1 (and later in the immunization experiments, tables 2, 3, and 4) shows that leukemogenic activity was absent or markedly decreased in several passages. Some mice appearing to be free from clinical leukemia for 6 months created the early impression that several fluids completely lacked leukemogenic activity (*1*). However, on prolonged observation of 8 to 10 months, lymphatic leukemias occurred in some of these mice. Table 1 also shows that "rejuvenation" increased the leukemogenic activity of the tissue culture fluids. It appears that after rejuvenation the number of characteristic reticulum cell leukemias with erythroblastosis but without thymic component especially increased. The appearance and proportion of reticulum cell and thymic (lymphatic) leukemias in mice inoculated with tissue culture materials, as related to the appearance of leukemias in mice inoculated with mouse passage line Rauscher virus, have been discussed earlier (*8*).

Histologically, some of the lymphatic leukemias showed sheets of lymphoid cells interspersed by numerous histiocytes. The histiocytes often were covered, surrounded, or penetrated by (phagocytized?) lymphocytes and reticulum cells (figs. 4, 5, and 6). A cell passage line of this lymphatic leukemia is now being maintained in mice and *in vitro*. A very similar "starry sky" appearance of lymph node sections was reported to be characteristic of Burkitt's human lymphoma (*9*).

In two 3-week-old female Timco Swiss mice inoculated with tissue culture fluid from passages 10 and 11, respectively, of line #3, very large intra-abdominal tumors developed 6 months later (fig. 7). At autopsy, the spleen, liver, thymus, and lymph nodes were not enlarged. Histologically, these tumors were mixed reticulum cell sarcomas and fibrosarcomas with foci of hematopoietic cells and round lymphoid cells. Primary tissue cultures of the tumors resulted in growth of the above-listed cell types (figs. 8, 9, and 10). The absence of gross leukemia in the tumor-bearing mice suggested that these tumors probably originated from tissue culture

TABLE 1.—Bioassays

Passage No.	Dilution of fluids and cells inoculated	Tissue culture lines#*					Types of leukemias†		
		1	2	3	4	Normal mouse embryo	Reticulum cell	Lymphoid	Reticulum cell sarcoma
1	10^0	2/7	4/8	10/11			9	1	
	10^{-2}	1/5							
2	10^0	2/6							
	10^{-2}	1/6							
3	10^0	0/5	3/3						
	10^{-2}	0/5							
4	10^0	0/6		4/6			4		
5	10^0		0/6						
6	10^0	0/10		2/6			2		
	Cells	0/9							
7	10^0			3/11				3	
8	10^0	1/28		0/5					
9	10^0			2/5			1	1	
10	10^0	0/10		2/5		0/21	1		1
11	10^0			6/12			2	3	1
12	10^0			8/8‡			6	2	
13	10^0			9/22			6	3	
15	10^0			2/7			2		
16	10^0			1/5	1/10			1	
17	10^0			0/5					
18	10^0			2/4			1	1	
19	10^0			0/5					
20	10^0			10/26‡			9	1	
21	10^0			1/4			1		
22	10^0			0/8					
28	10^0			1/10			1		
	Cells			0/9					
29	10^0			5/11‡			§		
	Cells			6/11			§		

*Leukemia/inoculated mice.

†Only in mice inoculated with line #3. Lines #1 and #2 caused reticulum cell leukemia.

‡Rejuvenated with normal fresh mouse embryo cells.

§Histology not complete. According to gross appearance, all leukemias so far are of reticulum cell type.

Cells = approximately 10^4 per mouse.

cells harvested accidentally when fluids were collected, and that these cells harbored leukemia virus without releasing it and had undergone malignant transformation and grew locally. Further observations on mouse and tissue culture passages of these cell lines will be reported separately.

Immunization Experiments

Three experiments will be reported here. In the first (table 2), 3-week-old Timco Swiss mice were pretreated with 0.1 ml of various tissue culture fluids. All of these fluids were stored deep frozen for several weeks. Three days and 3 weeks later, respectively, the mice were challenged by intraperitoneal inoculation of 0.1 ml Rauscher virus in the form of a leukemic stock spleen extract, diluted 10^{-2}. The results show that culture lines #3, carrying Rauscher virus, and #4, never intentionally infected with this virus, behaved alike. The degree of protection was barely significant, if the low leukemogenic potency of the challenging inoculum is considered in the control groups: 10 to 33% leukemia occurred in the experimental groups, as contrasted by 66 to 75% leukemia in the control groups. However, the incidence of leukemia in every experimental group was consequently lower than that in the control groups.

Table 3 shows another experiment, as it stands 5 months after its initiation. The intraperitoneal inoculation of tissue culture cells from line #3 protected the recipient mice somewhat against challenge with Rauscher virus, particularly when the interval between pretreatment and challenge was prolonged to 17 days. The fluid of the same cultures gave no protection against the challenge. This observation is well in line with the recent report of Barski and Youn (*3*).

Table 4 demonstrates that, in certain double-inoculated groups of mice, instead of protection, a somewhat accelerated form of leukemia may ensue. These mice first received low-leukemogenic tissue culture fluids and shortly afterward were challenged with leukemogenic Rauscher virus.

It is also important to note the low leukemogenic potency of the tissue culture fluids used for pretreatment in all these experiments.

Apparent protection of mice by the control cell line #4 (table 2) remains unexplained. Bioassays showed that, after 6 to 8 months' incubation, a few lymphatic leukemias may have developed in mice inoculated with fluids harvested from this line. These leukemias were not typed with the cytotoxic test at that time. It remains to be seen whether line #4 was accidentally contaminated with the Rauscher virus or with a mutant thereof, whether it harbors an unrelated leukemia virus, or whether it is free of leukemia virus after all. Should line #4 be found to carry a passenger mouse leukemia virus unrelated to Rauscher virus, then line #3 could possibly harbor both this agent and the Rauscher virus.

TABLE 2.—Immunization experiment with tissue culture fluids

Pretreatment and challenge	Passages used for pretreatment*										Total	Percent of leukemia
	3	4	5	8	9	10	11	13	17	24		
Line #1	0/5	0/5		0/9		0/5					0/24	0
Line #1 + RV,† 3 d‡	1/10	2/4				2/9					5/23	21
Line #1 + RV, 3 wk§	0/5					1/5					1/10	10
Line #2	0/5		1/7								1/12	8
Line #2 + RV, 3 d		3/9	1/5								4/14	28
Line #2 + RV, 3 wk	2/6										2/6	33
Line #3						1/4	0/7				1/11	9
Line #3 + RV, 3 d								2/8		2/7	4/15	26
Line #3 + RV, 3 wk						2/5			1/7		3/12	25
Line #4						1/8		0/4			1/12	8
Line #4 + RV, 3 d					2/7						2/7	28
Line #4 + RV, 3 wk					1/7			0/4			1/11	9
Normal ME‖						0/4					0/4	0
Normal ME + RV, 3 d					5/8	7/10					12/18	66
Normal ME + RV, 3 wk						3/4					3/4	75
Uninoculated											0/5	0
Uninoculated + RV, 3 d											6/8	75
Uninoculated + RV, 3 wk											5/7	71

*Leukemia/inoculated mice.

†RV = Rauscher virus.

‡3d = 3-day interval between pretreatment and challenge.

§3 wk = 3-week interval between pretreatment and challenge.

‖ME = mouse embryo tissue culture.

TABLE 3.—Immunization experiment with tissue culture cells

Material inoculated*		Interval between pretreatment and challenge (days)	Results	
Pretreatment	Challenge		Leukemia / Inoculated	% of leukemia
Cells, line #3, passage 25	RV†	17	3/19	15
”	RV	5	3/10	30
”	—	—	2/10	20
Fluid, line #3, passage 25	RV	17	8/9	88
Cells, normal mouse embryo tissue culture	RV	17	12/20	60
”	RV	5	8/9	88
”	—	—	0/10	0
Fluid, normal mouse embryo tissue culture	RV	17	5/5	100
”	—	—	0/5	0

*Approximately 10^4 cells per mouse were used; both pretreatment and challenge were given intraperitoneally. Fluid used for pretreatment was given intraperitoneally and was undiluted, 0.1 ml per mouse.

†RV = Rauscher virus extracted from leukemic mouse spleen.

TABLE 4.—Length of life of leukemic mice in certain doubly inoculated groups

Material inoculated		Interval between pretreatment and challenge (days)	Results		
				Average incubation of leukemia*	
Pretreatment (0.5 ml fluid/mouse)	Challenge		Leukemia / Inoculated	From pretreatment	From challenge
Line #2, passage 4	RV†	12	3/8	>131	>120
Line #2, passage 4	RV	1	8/8	55‡	54
Line #2, passage 4	—	—	2/8	>108	—
Line #4, passage 20	RV	12	6/6	>96	>84
Line #4, passage 20	RV	1	3/3	76§	75
Line #4, passage 20	—	—	0/6	0	0
None	RV	—	3/3	—	110

*From inoculation to death, in days; where some of the leukemic mice are still alive, figures were marked with >.

†RV = Rauscher virus extracted from leukemic mouse spleen.

‡Deaths of individual mice: on day 20, 29, 30, 35, 61, 70, 75, and 124.

§Deaths of individual mice: on day 51, 84, and 95.

Cytotoxic Antibody Determinations

These tests were done with sera pooled per groups from surviving mice that were killed 7 months after pretreatment with tissue culture fluids and challenge with mouse-passaged Rauscher virus (these mice were shown in table 2). Table 5 demonstrates that no cytotoxic antibodies could be found in those mice receiving only tissue culture fluids, although some of these fluids were able to protect other groups of mice against challenge with leukemogenic virus. A low but readily detectable level of cytotoxic antibodies was found in several groups of mice that were both pretreated and challenged but remained free from leukemia. Presumably, these mice received two antigenic stimuli: the first at pretreatment and the second

Table 5.—Cytotoxic tests

Groups of mice from which sera* were derived		% of diffusely pink, swollen cells
Pretreatment with tissue culture line	Challenge	
#1, passage 3	RV,[†] 3 d[‡]	13
#1, passage 3	RV, 3 w[§]	6
#1, passage 3	—	1
#1, passage 4	RV, 3 d	10
#1, passage 4	—	1
#1, passage 8	—	2
#1, passage 10	RV, 3 d	8
#1, passage 10	RV, 3 w	5
#2, passage 3	RV, 3 w	6
#2, passage 3	—	1
#2, passage 4	RV, 3 d	9
#2, passage 5	RV, 3 d	2
#2, passage 5	—	1
#3, passage 10	RV, 3 w	9
#3, passage 10	—	<1
#3, passage 11	—	5
#3, passage 13	RV, 3 d	12
#3, passage 17	RV, 3 w	12
#3, passage 24	RV, 3 d	15
#4, passage 9	RV, 3 d	3
#4, passage 10	RV, 3 w	1
#4, passage 10	—	1
#4, passage 13	RV, 3 w	2
Control, mouse embryo tissue culture	RV, 3 d	2
Control, mouse embryo tissue culture	RV, 3 w	5
—	RV	3
—	—	2

*Pooled per groups; obtained 7 months after pretreatment and challenge; used as 1:4 dilution.

[†] RV = Rauscher virus extracted from leukemic mouse spleen.

[‡] 3 d = 3-day interval between pretreatment and challenge.

[§] 3 w = 3-week interval between pretreatment and challenge.

at challenge. In this study, line #4 acted as if it contained no specific Rauscher virus antigen, for no cytotoxic antibodies were detectable in mice that were pretreated only, nor in those which were both pretreated and challenged. It is possible, however, that these mice circulated some cytotoxic antibodies earlier. The presence of cytotoxic antibodies could not be correlated readily with leukemic cagemates: Both absence and presence of these antibodies may have occurred in pooled sera of mice who were caged for several months with leukemic mates.

Interferon Assay and Direct Interference

Table 6 shows that no interferon-like inhibitor could be demonstrated with certainty in culture fluids of advanced passages of line #3, for the cytopathic effect and yield of vesicular stomatitis or pseudorabies viruses in control or experimental cultures were almost identical. Especially little difference in the above parameters (cytopathic effect and virus yield) occurred when pretreatment was limited to 6 hours. In cultures pretreated for 30 hours before challenge, some, perhaps not interferon mediated, interference might have occurred. The positive interferon control preparation inhibited both the cytopathic effect and multiplication of vesicular stomatitis virus. The cytopathic effect of the pseudorabies virus showed little sensitivity to this preparation. However, the yield of infectious pseudorabies virus particles was inhibited in cultures pretreated with this preparation, as shown by retitration of the infectious virus yield. This suggests that the mechanism of the cytopathic effect of these two viruses is different, *i.e.*, for the cytopathic effect of the pseudorabies virus, the formation of complete, fully infectious virus is not essential.

Studies on direct interference revealed nothing. Two experiments were carried out. With the use of 8 cultures per dilution for the retitration of vesicular stomatitis virus, it was found that 3 times and 17 times less virus could be recovered from cells of line #3 than from cells of line #4, both of which were infected with approximately equal multi-

TABLE 6.—Interferon assay

		Early results†		Late results‡	
Pretreatment*	Challenge	CP§ (%)	Titer‖	CP§ (%)	Titer‖
Line #4	VSV	75	−3.5	65	−4.0
"	PRV	90	−1.8	83	−2.7
Normal mouse serum	VSV	100	−3.3	65	−4.0
"	PRV	83	−1.8	75	−2.7
Interferon-containing serum	VSV	8	−1.5	40	−2.5
"	PRV	65	−0.5	65	−2.0
Line #3, passage 8	VSV	75	—	83	—
"	PRV	83	—	58	—
Line #3, passage 9	VSV	75	—	65	−4.5
"	PRV	83	—	33	−2.0
Line #3, passage 20	VSV	83	−3.0	33	−2.5
"	PRV	65	−2.0	50	−2.0
Line #2, passage 4	VSV	83	>−2.8	33	−2.9
"	PRV	50	−2.0	33	−1.9
Tissue culture medium, control	VSV	83	−3.3	75	−4.3
"	PRV	65	−2.0	83	−2.4

*Tissue culture fluids used for pretreatment of mouse embryo monolayers before challenge with vesicular stomatitis (VSV) or pseudorabies virus (PRV).

†Pretreatment before challenge for 6 hours.

‡Pretreatment before challenge for 30 hours.

§Extent of cytopathic effect, in estimated % of surface area of culture.

‖Titer of challenging viruses (VSV or PRV, respectively) as harvested from assay cultures (pooled per groups).

plicities of this virus. Despite this slight difference in virus yield, which could be ascribed to minor errors of the multiplicities of the infection, the cytopathic effect of the vesicular stomatitis virus in both lines #3 and #4 was practically identical.

It remains disputable whether the slight decrease in the yield of vesicular stomatitis virus from normal mouse embryo cultures pretreated with leukemic tissue culture fluids (line #3, passage 20, and line #2, passage 4) for 30 hours (table 6) could be attributed to direct interference between leukemia virus and vesicular stomatitis virus.

Other Studies in Progress

When trypsinized cells of line #3, passage 35, were suspended and exposed to cytotoxic mouse serum in the presence of guinea pig complement and eosin, only 10% of the cells responded with slight swelling and pinkish discoloration. Control tissue culture cells that were not supposed to carry the Rauscher virus, such as healthy mouse embryo cultures or cells of line #4, failed to show cytotoxic effect in this test. Further studies are needed to perfect this procedure.

A possible thermoinactivation of Rauscher virus kept at 37° C is being investigated. When Rauscher virus in the form of a 10% cell-free leukemic spleen extract was kept in sealed glass containers at 37° C for a prolonged time, samples taken at zero hour caused leukemia readily, but samples withdrawn 4 and 7 days later failed to cause leukemia in mice within 4 months.

Dr. J. A. Sykes maintains our lines #3 and #4 from passages 35 and 27, respectively, in his laboratory. In a preliminary study, Dr. L. Dmochowski (personal communication) prepared ultrathin sections from embedded cells of line #3 and recognized a great many typical type C mouse leukemia virus particles. The Rauscher virus-carrying tissue culture line of Wright and Lasfargues also contains predominantly type C particles (*2*).

DISCUSSION

These studies suggest that replicating but weakly leukemogenic Rauscher virus may persist in a serially passaged tissue culture line. The crucial question is whether this change in the virus is merely quantitative or truly qualitative, *i.e.*, attenuation. Attenuation would mean that a large amount of virus particles, each with decreased leukemogenic potency, is manufactured. However, it has not yet been determined how many leukemia virus particles form one LD50 in the mouse; it may well be that thousands of particles are needed to initiate leukemogenesis. Thus a second possibility emerges, namely, that fibroblastic and other tissue culture cells replicate only a few virus particles per cell, and if only a small proportion of the total cell population is involved in virus replication, an immunizing but not leukemogenic dose of virus particles may only be recovered from the cultures and delivered to individual mice in the bioassay. In other words, the subliminal dose of the virus is responsible for the failure to cause leukemia in mice, rather than a qualitative change (attenuation) of the individual virus particles. A third possibility is that some extracellular virus particles in the tissue culture fluid may undergo slow heat inactivation at 37° C in a few days, and may exert immunogenic but not leukemogenic action. Our studies do not clearly distinguish as yet between these possibilities. In our immunization experiments, smaller volumes of tissue culture fluids (0.1 ml) were used for pretreatment than those tested for leukemogenic potency in the bioassays (0.5 ml); thus a subliminal but immunizing dose of virus could be responsible for the effects described. Among other tests, electron microscopic studies as to number and structure of particles present are now essential.

Even in the case of true attenuation, the virus population still must consist of at least two types of particles: the attenuated particles and a smaller amount of fully leukemogenic particles. Addition of fresh mouse embryo cells to the culture would promote the replication of the fully leukemogenic particles, whereas in later passages fibroblastic cells would support again the growth of the attenuated particles. This hypothesis is aimed to explain the phenomenon of "rejuvenation."

If challenge with leukemogenic virus particles extracted from spleens of leukemic mice follows the inoculation of attenuated particles 3 weeks later, full immunity may be observed. However, when the interval between pretreatment with attenuated virus and challenge with leukemogenic virus is only a few days, merely an equivocal degree of protection can be observed. It appears that in some cases an accelerated form of leukemia occurs under such circumstances. This phenomenon suggests that the attenuated particles could utilize part of the genome of the leukemogenic particles, and incorporation of these presumably ribonucleic acid fragments into the attenuated particles would restore their leukemogenic potency with resulting overwhelming viremia and rapid splenomegaly and finally, early death.

SUMMARY

The Rauscher mouse leukemia virus has been maintained in normal Swiss mouse embryo cells that were cultured *in vitro* and passaged 42 times within 14 months. Decreased leukemogenic activity was detected by intraperitoneal inoculation of 3-week-old Swiss mice after several passages of the virus carrier cells. Increased leukemogenic activity of the tissue culture fluids of later passages reappeared after "rejuvenation" of the cultures by the addition of fresh healthy mouse embryo cells. Interferon

does not appear to play a significant role in the suppression of the leukemogenic activity of the Rauscher virus in these cultures. Young Swiss mice, pretreated with low leukemogenic tissue culture fluids or cells, developed leukemia only in low incidence after challenge 3 weeks later with highly leukemogenic (leukemic mouse spleen extract) Rauscher virus. However, in certain similarly pretreated groups challenged with leukemogenic virus 1 day after pretreatment, accelerated leukemia occurred. Possibly attenuation of Rauscher virus occurs during propagation *in vitro*. Advanced passages of such cultures may contain a mixture of nonleukemogenic and leukemogenic virus particles. Fresh mouse embryo cells may promote the replication of leukemogenic particles, whereas in aging fibroblastic cultures low leukemogenic particles may prevail. Inoculation of both forms of particles into mice may result in interference, accelerated leukemia, or immunization, depending on presently incompletely understood proportion and sequence of these inocula.

REFERENCES

(*1*) Sinkovics, J. G., Bertin, B. A., and Howe, C. D.: Latency of mouse leukemia virus in tissue cultures as related to cell growth and interferon. Abstract of paper presented at the Sixteenth Annual Meeting of the Tissue Culture Association, Miami Beach, Fla., 1965, p 21.

(*2*) Wright, B. S., and Lasfargues, J. C.: Long-term propagation of the Rauscher murine leukemia virus in tissue culture. J Nat Cancer Inst 35: 319–327, 1965.

(*3*) Barski, G., and Youn, J. K.: Immunization against Rauscher mouse leukemia with tissue culture material. Science 149: 751–752, 1965.

(*4*) Merchant, J. L., Kahn, R. H., and Murphy, W. H.: Handbook of Cell and Organ Culture. Minneapolis, Burgess Pub. Co., 1960.

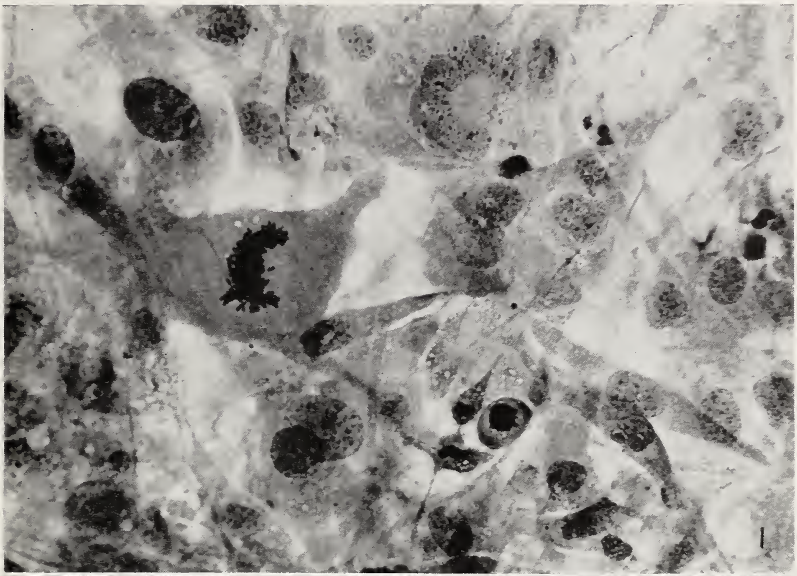
(*5*) Reed, L. J., and Muench, H.: A simple method of estimating 50 per cent endpoints. Amer J Hyg 27: 493–497, 1938.

(*6*) Baron, S., and Buckler, C. E.: Circulating interferon in mice after intravenous injection of virus. Science 141: 1061–1063, 1963.

(*7*) Sinkovics, J.: Interactions of the Newcastle disease virus with mouse tissues. Arch Virusforsch 10: 103–125, 1960.

(*8*) Sinkovics, J. G., and Howe, C. D.: Growth characteristics, virus yield and interferon assay of leukemic mouse spleen tissue cultures. J Infect Dis 114: 359–372, 1964.

(*9*) Dorfman, R. F.: Childhood lymphosarcoma in St. Louis, Missouri, clinically and histologically resembling Burkitt's tumor. Cancer 18: 418–430, 1965.



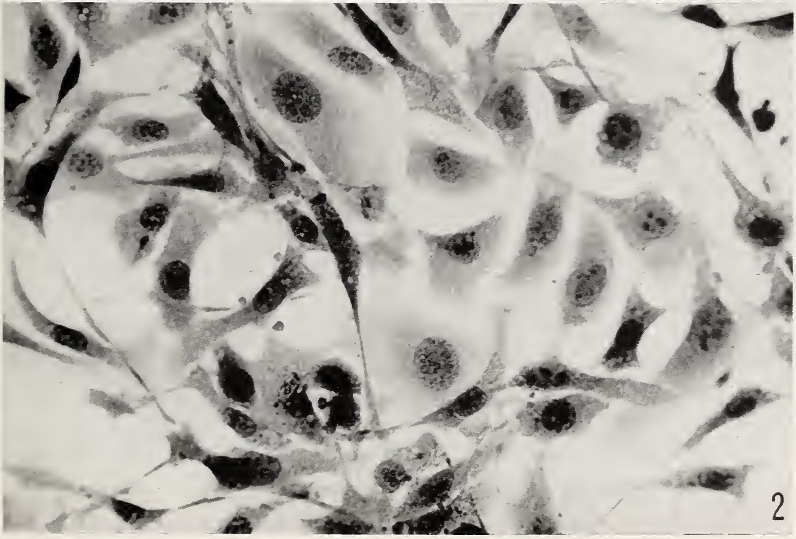


Figure 1.—Multilayered growth of line #3 in cell passage 35. Wright stain from Leighton tube. × 375

Figure 2.—Growth pattern of line #3 in cell passage 41. Wright stain from Leighton tube. × 375

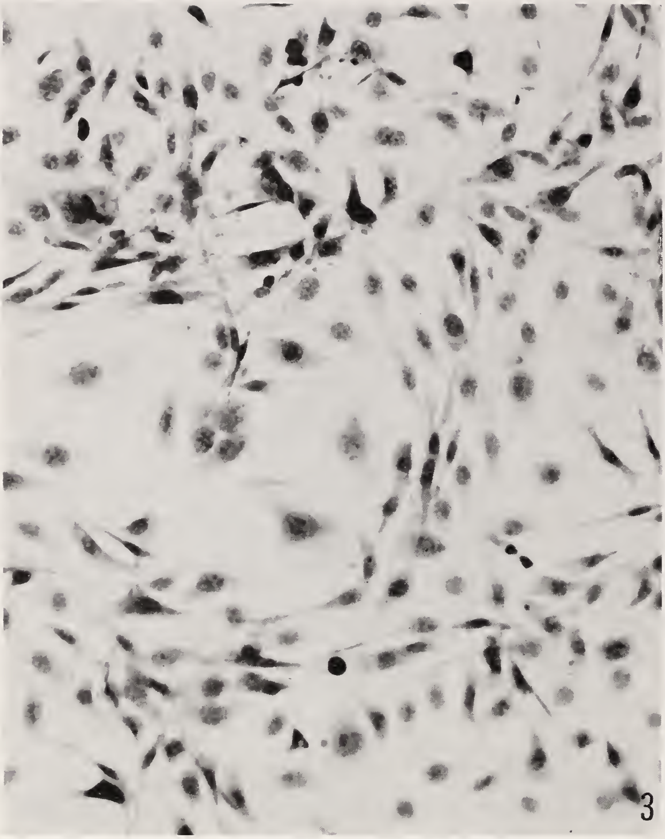
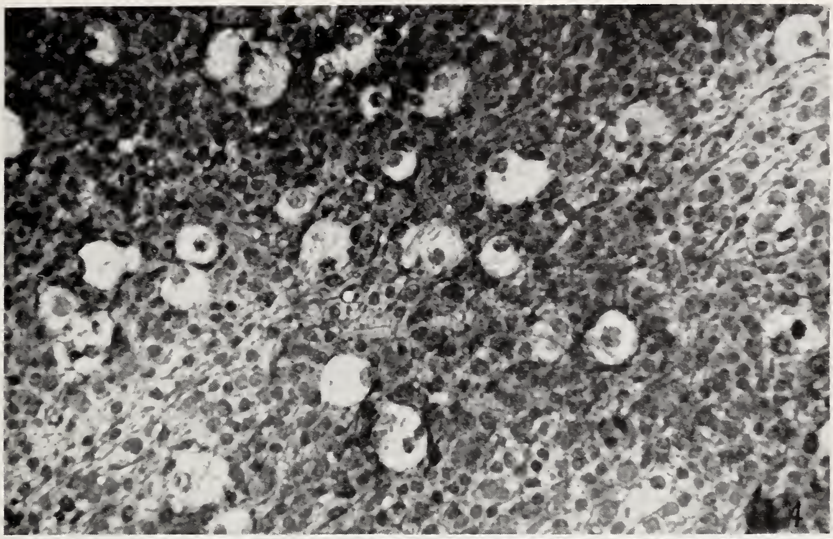
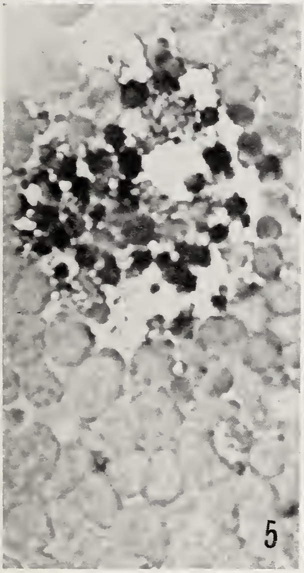


FIGURE 3.—Growth pattern of line #4 in passage 33. Wright stain from Leighton tube. × 300





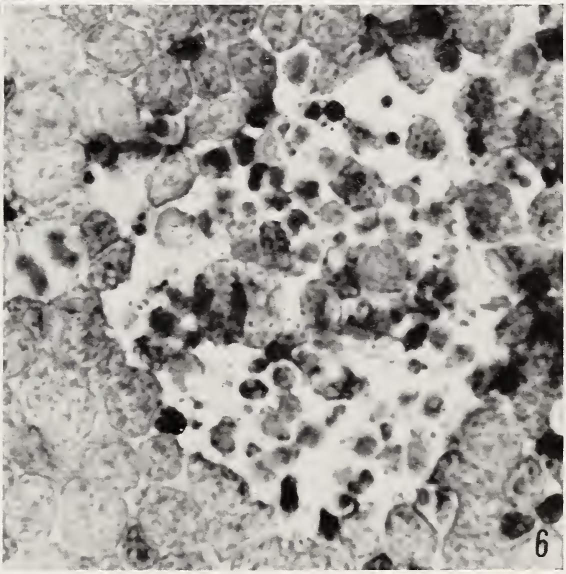
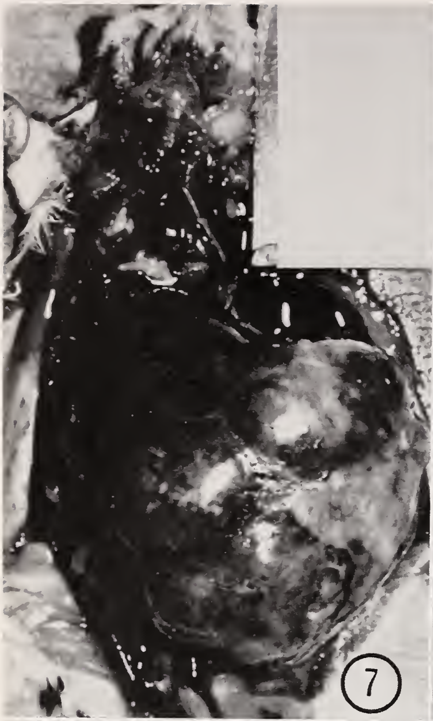
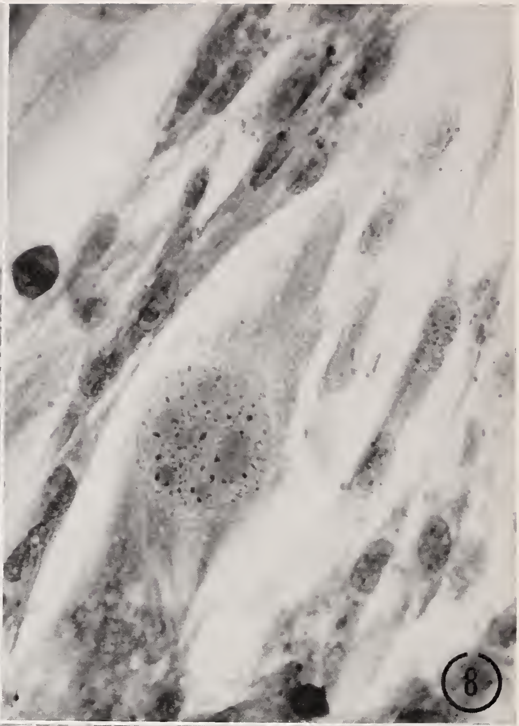


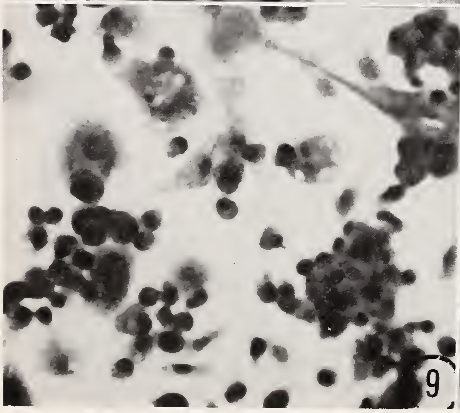
Figure 4.—"Starry sky" appearance of mouse lymphoma. Sheets of lymphoblasts are interspersed by histiocytes. Print preparation, Wright stain. × 300

Figure 5.—A histiocyte covered by small lymphocytes or by pyknotic nuclei of other dead cells. Print preparation from mouse lymphoma, Wright stain. × 375

Figure 6.—Cell debris in cytoplasm of a histiocyte. Print preparation from mouse lymphoma, Wright stain. × 720







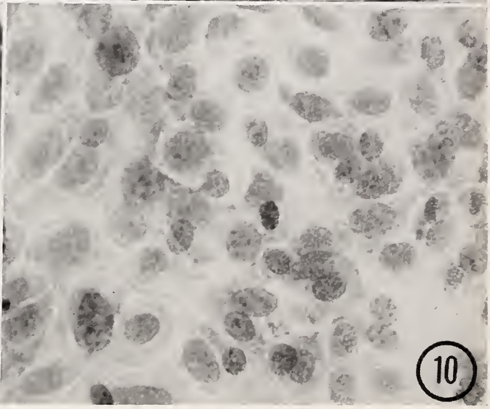


FIGURE 7.—Mouse with a huge mixed reticulum cell sarcoma and fibrosarcoma.

FIGURE 8.—Growth of fibroblast-like cells *in vitro* from the tumor shown in figure 7. Wright stain from Leighton tube. × 375

FIGURE 9.—Clumps of lymphocytes, plasma cells, and macrophages as seen in the primary tissue culture of the tumor shown in figure 7. Wright stain from Leighton tube. × 300

FIGURE 10.—Sheet of small cuboid cells, resembling reticulum cells, growing from primary explants of the tumor shown in figure 7. Wright stain from Leighton tube. × 300

DISCUSSION

Dr. Barski: I want to ask Dr. Sinkovics about the rejuvenation procedure: Why did you find it necessary? Was cell growth arrested? On the other hand, what were the modifications of the cell morphology after the rejuvenation?

Dr. Sinkovics: I felt it necessary because the culture seemed to die out. When we transferred it, we lost 75% of the cells. They spread out but failed to divide, or they didn't even spread out. So we were afraid we would lose the culture and that is why we mixed it with fresh cells.

The morphology after rejuvenation appeared as that of a new culture: There were undifferentiated mesenchymal cells interspersed by fibroblasts. After 1 or 2 passages the undifferentiated cells were lost and then two cell types remained: a fibroblast and a reticulum cell. And this is how it looks today.

Dr. Barski: Did you have the same phenomenon in the parallel noninfected line?

Dr. Sinkovics: Yes. The noninfected line was also on a downhill course. We rejuvenated it twice, with the same type of cells, and both cultures grow well today. I don't think they will have to ever be rejuvenated again; it is easy to handle them now.

Infective and Noninfective Viral Murine Leukemias [1,2,3]

W. H. Kirsten,[4] L. A. Mayer, *and* C. W. Welander,[5] *Departments of Pathology and Pediatrics, Division of Biological Sciences, The University of Chicago, Chicago, Illinois*

Evidence from experiments with Rous sarcoma virus and the virus of avian myeloblastosis indicates that RNA tumor viruses are the direct cause of neoplasia not only by initiation of cancer but also by subsequent release of virus from the tumor cells and neoplastic transformation of new target cells in the tumor-bearing host (*1–3*). According to Bryan (*4*), such tumors are recognized as virus-dependent reactions. The marked viremia during murine leukemogenesis and other parameters of murine leukemias suggest that they may belong to the same category of neoplasia (*4–6*). The role of virus release in the progression of leukemia from an initial focus of transformed cells to a systemic disease is difficult to demonstrate by *in vivo* experiments. Tissue culture of virus-induced murine leukemic cells provides an experimental model which allows for the comparison of virus release from various lines of murine leukemic cells. This report describes infective and noninfective murine leukemic cell lines. Virus release from these cells is determined by bioassays of culture supernatants, transplantation, and electron microscopy.

MATERIALS AND METHODS

Leukemia virus.—The Passage A virus of Gross was transmitted to noninbred Wistar rats and to inbred Wistar/Furth (W/Fu) rats (*7*, *8*). Plasma pellets were prepared by differential centrifugation (*9*), suspended

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported by Public Health Service research grant R1 CA-4311 from the National Cancer Institute.

[3] For convenience, the terms "leukemia" and "lymphoma" are used as synonyms in this paper.

[4] U.S. Public Health Service Career Development Awardee.

[5] We wish to acknowledge the excellent technical assistance of Mrs. F. Cardenas.

in 1 ml of Dulbecco's phosphate-buffered saline (PBS), and inoculated into 19 neonatal W/Fu rats. All recipients developed thymic lymphomas 8 to 11 weeks later. The tissue cultures derived from these lymphomas were designated "high-dose leukemic cell lines." In contrast, "low-dose leukemic cell lines" referred to thymic lymphomas induced in 2 of 34 W/Fu rats after 26.6 weeks by a 10^{-8} dilution of the same plasma pellet.

Tissue culture.—Fragments of viable thymic tumors were removed under aseptic conditions, washed in 3 changes of Hanks' balanced salt solution, and minced with scissors. The resulting brei was washed again and suspended in 0.25% trypsin at 37° C in a magnetic stirrer. Following trypsinization and straining, the cells were washed twice and suspended in Puck's medium N16 or medium 199, 20% fetal bovine serum, 2% glutamine, and antibiotics. Two-ounce prescription bottles were seeded with 10^5 and 10^6 cells per ml and incubated at 37° C under constant flow of 5% CO_2 in air. The medium was changed twice weekly. Subcultures were made by trypsinization or scraping with a rubber policeman. The monolayer cultures from the thymic lymphomas used for these studies were maintained for 8 to 9 weeks *in vitro*. Culture supernatants were collected 2 to 3 days after the last medium change and centrifuged at 6550 × *g* for 10 minutes at 4° C. The supernatant was pipetted off and centrifuged at 104,000 × *g* for 1 hour at 4° C. The resulting pellet was prepared as described above.

Rats.—The origin and maintenance of the inbred strains of Wistar/Furth (W/Fu) and August (A) rats were described (*10*).

Electron microscopy.—Tissue culture cells were loosened from the glass surface by glass beads, centrifuged at low speed, fixed for 1 hour in 1% phosphate, or Veronal-buffered OsO_4, dehydrated in a graded series of ice-cold ethanol solution, and embedded in Epon. Sections were stained in freshly prepared 50% ethanol saturated with uranyl acetate for 10 to 30 minutes and examined in a Hitachi HU 11 A electron microscope at 50 kv accelerating voltage.

RESULTS

Morphologic Characteristics of Leukemic Cell Cultures

Monolayer cultures from lymphomas of W/Fu rats consisted of reticular cells and round, lymphoblast-like cells (*11*, *12*). This symbiotic growth pattern persisted for 3 to 4 subcultures, but the number of lymphoblast-like cells disappeared with increasing age of the cultures. After 6 to 7 subcultures, only reticular cells forming a continuous monolayer were present. More recent observations suggested that substitution of Puck's N-16 medium by medium 199 and fetal bovine serum restored the growth of lymphoblast-like cells, particularly at bovine-serum concentrations of

20%. We could not determine whether these lymphoblast-like cells multiplied inside the reticular cells as suggested by Ioachim and Furth (*11*), although "emperiopolesis" was occasionally observed in the cultures.

Transplantability of Leukemic Cells Grown *In Vitro*

Pilot experiments established that thymic lymphoma cells from W/Fu rats, propagated *in vitro* for periods of up to 8 weeks, were transplantable to weanling W/Fu rats but not to homologous A rats of similar age. All inoculations were intraperitoneal, and cell dosages ranged from 10^4 to 30×10^6 viable cells per rat.

Cells from the high-dose leukemic cell line were transplantable to weanling and neonatal W/Fu rats but not to weanling A rats (table 1). Thirteen of 14 A rats inoculated at birth apparently rejected the leukemic-cell grafts. Nine of these 13 rats (70%) died from thymic lymphosarcomas after 17 to 22 weeks. Transplants from the thymic lymphosarcomas in A rats were accepted by adult A, but not by W/Fu rats. The difference between transplanted and virus-induced leukemias was morphologically striking (*8*, *10*, *12*, *13*). Transplanted leukemias metastasized from the abdominal cavity to distant organs, including the parathymic lymph nodes (*12*), but did not involve the thymus, whereas virus-induced leukemias were invariably thymic in origin. The massive intra-abdominal tumor growth, so characteristic of leukemia transplants, was not observed in thymic lymphomas.

Cells from the low-dose leukemic cell lines grew only in isologous W/Fu rats. Furthermore, virus-induced leukemias were not observed after 1 year in any of the A rats that received as newborns cell inocula of the same quantity as did A rats injected with the high-dose cell line.

TABLE 1.—Transplantability of high-dose and low-dose leukemic cell lines derived from thymic lymphomas of W/Fu rats

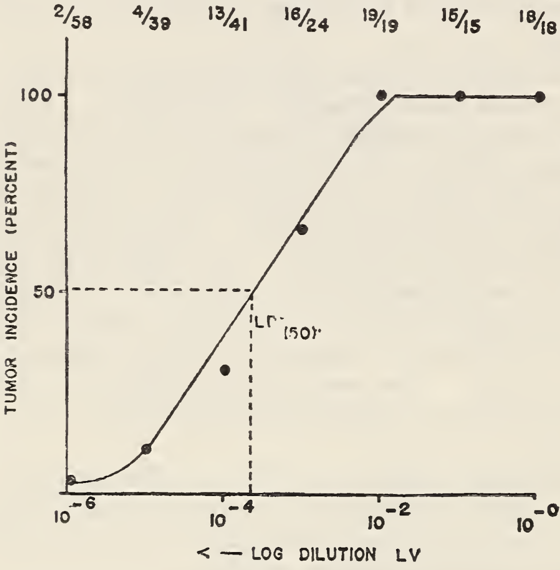
		Type of leukemia†			
		Transplanted		Virus induced	
Type of leukemic cell line	Recipients*	Number leukemic / Number injected	Latent period (weeks)	Number leukemic / Number injected	Latent period (weeks)
High dose	W/Fu Weanling	10/10	3	—	—
	W/Fu Newborn	12/12	2	—	—
	A Weanling	0/17	—	0/17	—
	A Newborn	1/14	4	9/13	17–22
Low dose	W/Fu Weanling	9/10	2–6	—	—
	W/Fu Newborn	12/12	3	—	—
	A Weanling	0/15	—	0/15	—
	A Newborn	0/14	—	0/14	—

*Weanling rats were between 29 and 37 days old; newborn rats were 2 to 3 days old.

†Viable leukemic cells (*10*) were given intraperitoneally to the recipients in doses of 8×10^4 to 3×10^6 cells per rat.

Bioassays of Culture Supernatants

Pellets from the culture supernatant fluids were prepared after 7 weeks of cultivation, with the exception of one low-dose cell line which was harvested for bioassays after 5 weeks *in vitro*. Tenfold serial dilutions (10^{-1} to 10^{-6}) were prepared in PBS before inoculation into neonatal W/Fu rats. Each rat received 0.2 ml of the virus material intraperitoneally. The results of bioassays from 3 different high-dose cell lines are shown in text-figure 1.



TEXT-FIGURE 1.—Leukemia incidence in W/Fu rats after inoculation of leukemia virus obtained from the pelletized culture supernatants of the high-dose leukemic cell cultures.

Biologically active leukemia virus was recovered from the pellets of the high-dose leukemic cell cultures. The initiating dose was of primary importance in determining the leukemia incidence. Compilation of the mean latent periods for leukemias revealed no relationship between infecting virus dose and the time elapsing from virus inoculation to death. This was attributable to variations in the growth patterns of some virus-induced thymic lymphomas. Most rats died from large thymic lymphomas with metastases to the sternal bone marrow, lungs, spleen, liver, and lymph nodes. Small thymic lymphomas extending primarily into the posterior mediastinum were observed in approximately 15% of the rats. Frequently, rats with lymphoma involvement of the spinal cord died after a relatively short latent period from paralysis of the extremities. Since such rats belonged to various dilution groups (including the 10^{-5} dilution), the evaluation of the latent periods relating to the infecting virus dose would have been heavily biased by the peculiar growth pattern of these lymphomas.

Culture supernatants from the low-dose leukemic cell lines were prepared as dilutions 10^{0}, 10^{-1}, and 10^{-2}. A total of 49 W/Fu rats were inoculated; none had developed thymic lymphoma after over 12 months' observation. Although animal inoculations are relatively insensitive assay methods, these negative results suggest that these cell lines do not release biologically active virus.

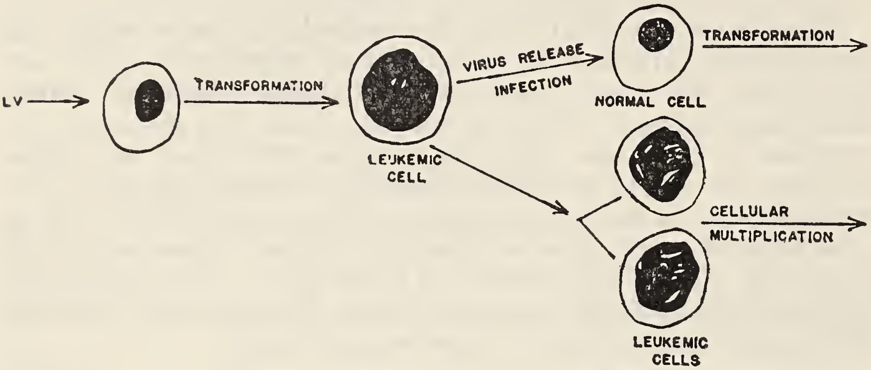
Electron Microscopy

Ultrathin sections from the high-dose leukemic cell lines revealed many leukemia-virus particles in the pelletized tissue culture fluids and between the leukemic cells after 3, 5, and 8 weeks of *in vitro* propagation. Membrane-bound aggregates of murine leukemia viruses were also seen in the cytoplasm of some cells. The virus particles (75–110 mμ) were of the doughnut type and of the "mature" type with nucleoids (*14–17*) (fig. 1). The first assays of the low-dose leukemic cell lines after 3 and 5 weeks *in vitro* failed to reveal leukemia viruses in 30 sections. However, an occasional intercellular virus particle was found after 7 to 8 weeks (fig. 2), suggesting that the low-dose leukemic cell lines were not entirely virus-free. Electron microscopic examinations are being continued.

DISCUSSION

The data presented here are significant only in that they may lead to further experiments on the role of virus release from leukemic cells in murine leukemogenesis. From pathogenetic studies it is generally assumed that viral lymphomas and leukemias progress by cellular multiplication and metastases. These detailed histologic analyses are presumably derived from experiments in which large doses of leukemia virus are applied to obtain a positive response (*18–22*). The present studies illustrate that high-dose leukemic cell lines can continue growth *in vitro* (*12*) and release appreciable amounts of infectious virus progeny. The tissue-cultured leukemic cells retain their specific transplantation antigens after 8 weeks of *in vitro* propagation, and as transplanted cells release infectious virus as judged from the development of virus-induced lymphomas in neonatally inoculated homologous hosts (table 1). A similar phenomenon of early or grafted and late or virus-induced leukemias has been previously observed after heterotransplantation of lymphoma cells from AKR mice to newborn rats (*10*). The two leukemic manifestations are distinguishable by differences in the sites of origin, latent periods, antigens of their respective tumor cells, and species-specific chromosomes (*13*).

To our knowledge, no information is available on the fate of microscopic foci of leukemic or lymphoma cells which follow the *in vivo* inoculation of very low doses of leukemia virus. If the *in vivo* situation is indeed comparable to the low-dose leukemic cell cultures, virus release from such cells is quantitatively below the leukemogenic level. The low-dose leukemic cell lines are considered noninfective rather than virus-free (fig. 2). Does the negative response following animal inoculation of low doses of leukemia virus imply regression of an initial tumor focus? Is the tumor rejected because of tumor-specific antigen(s) (*23*, *24*), or rising titers of virus neutralizing antibodies, or failure to release virus? Is virus release essential for continued growth of viral lymphomas and leukemias as proposed in text-figure 2? According to this concept, viral murine leukemias produce a dual type of disease (*25*). The initial transformation of normal to leukemic cells is followed by production of mature particles, infection, and subsequent leukemic transformation of new target cells. The role of such a two-stage mechanism has yet to be elucidated in the natural host; its importance is obvious in attempts to isolate similar agents from leukemias of unknown etiology.



TEXT-FIGURE 2.—Progression of viral murine leukemias by cellular multiplication and infection of new target cells.

SUMMARY

Thymic lymphomas were induced in W/Fu rats by high and low doses of leukemia virus (Gross) and grown as monolayer cultures for 8 weeks. High-dose leukemic cell cultures were infective as judged from bioassays, electron microscopy, and induction of thymic lymphomas in homologous A rats following cell transplants. Low-dose leukemic lines were noninfective. The role of virus release from leukemic cells in leukemogenesis is discussed.

REFERENCES

(*1*) BALUDA, M. A.: Properties of cells infected with avian myeloblastosis virus. *In* Basic Mechanisms in Animal Virus Biology. Sympos Quant Biol 27: 415–425, 1962.

(*2*) PONTÉN, J.: Homologous transfer of Rous sarcoma by cells. J Nat Cancer Inst 29: 1147–1159, 1962.

(*3*) BERGS, V. V. and GROUPÉ, V.: Low malignancy of Rous sarcoma cells as evidenced by poor transplantability in turkeys. Science 139: 922–923, 1963.

(*4*) Bryan, W. R.: Some biological considerations of tumor viruses. *In* Symposium on Cellular Control Mechanisms and Cancer (Emmelot, P., and Mühlbock, O., eds.). Amsterdam, Elsevier Publ Co., 1964, pp 338–355.

(*5*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*6*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*7*) Gross, L.: Pathogenic potency and host range of the mouse leukemia virus. Acta Haemat 29: 1–15, 1963.

(*8*) Kirsten, W. H., Platz, C. E., and Flocks, J. S.: Lymphoid neoplasms in rats after inoculation with cell-free extracts of leukemic AKR mice. J Nat Cancer Inst 29: 293–319, 1962.

(*9*) Okano, H., Kunii, A., and Furth, J.: An electron microscopic study of leukemia induced in rats with Gross virus. Cancer Res 23: 1169–1175, 1963.

(*10*) Kirsten, W. H., and Platz, C. E.: Early and late leukemias in rats after transplantation of leukemic cells from AKR mice. Cancer Res 24: 1056–1062, 1964.

(*11*) Ioachim, H., and Furth, J.: Intrareticular cell multiplication of leukemic lymphoblasts in thymic tissue cultures. J Nat Cancer Inst 32: 339–359, 1964.

(*12*) Ioachim, H. L., Cali, A., and Sinha, D.: Age-dependent transplantability in rats of virus-induced thymic lymphoma cultured *in vitro*. Cancer Res 25: 132–139, 1965.

(*13*) Kirsten, W. H., and Dominguez, R. H.: Chromosome studies on transplanted and virus-induced lymphoid leukemias in rats. Cancer Res 26: 816–821, 1966.

(*14*) Bernhard, W.: The detection and study of tumor viruses with the electron microscope. Cancer Res 20: 712–727, 1960.

(*15*) Dalton, A. J.: Micromorphology of murine tumor viruses and of affected cells. Fed Proc 21: 936–941, 1962.

(*16*) Dmochowski, L., Gross, L., and Padgett, F.: Electron microscopic studies of rat leukemia induced with mouse leukemia virus. Proc Soc Exp Biol Med 110: 504–508, 1962.

(*17*) Feldman, D. G., and Gross, L.: Electronmicroscopic study of the mouse leukemia virus (Gross), and of tissues from mice with virus-induced leukemia. Cancer Res 24: 1760–1783, 1964.

(*18*) Metcalf, D., Furth, J., and Buffett, R. F.: Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res 19: 52–58, 1959.

(*19*) Dunn, T. B., Moloney, J. B., Green, A. W., and Arnold, B.: Pathogenesis of a virus-induced leukemia in mice. J Nat Cancer Inst 26: 189–221, 1961.

(*20*) Levinthal, J. D., Buffett, R. F., and Furth, J.: Pathogenesis of mouse viral leukemia. Proc Soc Exp Biol Med 106: 455–459, 1961.

(*21*) Siegler, R., Geldner, J., and Rich, M. A.: Histogenesis of thymic lymphoma induced by a murine leukemia virus (Rich). Cancer Res 24: 444–459, 1964.

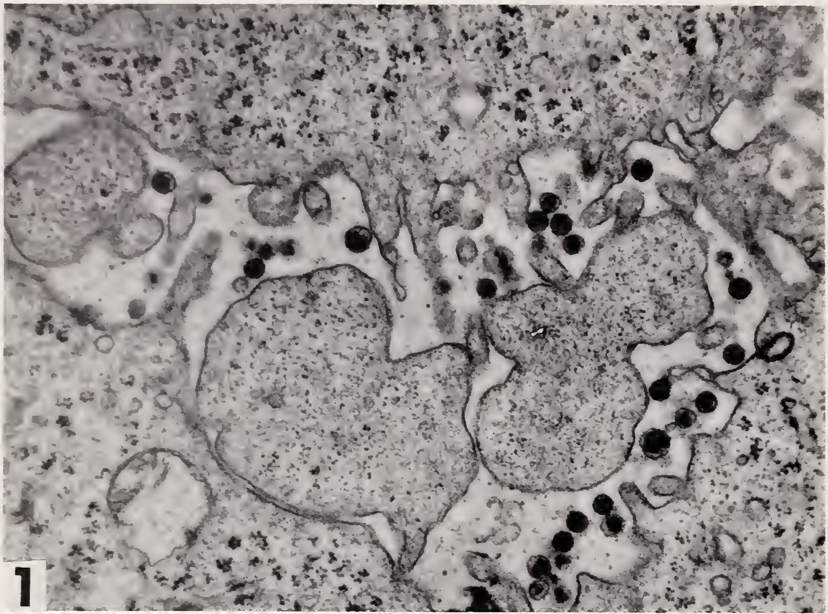
(*22*) Siegler, R., and Rich, M. A.: Comparative pathogenesis of murine viral lymphoma. Cancer Res 24: 1406–1417, 1964.

(*23*) Klein, G., Sjögren, H. O., and Klein, E.: Demonstration of host resistance against isotransplantation of lymphomas induced by the Gross agent. Cancer Res 22: 955–961, 1962.

(*24*) Rubin, H.: The immunological basis for non-infective Rous sarcomas. *In* Basic Mechanisms in Animal Virus Biology. Sympos Quant Biol 27: 441–452, 1962.

(*25*) Bryan, W. R.: Viral etiology of leukemia. Combined clinical staff conferences at the National Institutes of Health. Ann Intern Med 62: 376–399, 1965.

MURINE LEUKEMIA



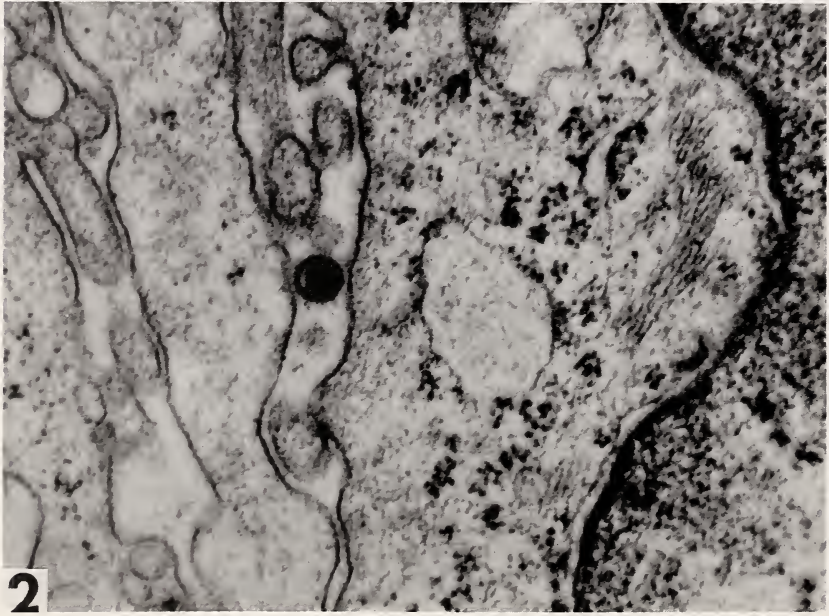


FIGURE 1.—Section through a pellet obtained from a cell culture after 8 weeks of *in vitro* propagation. Multiple leukemia-virus particles are seen in intercellular space. × 35,000

FIGURE 2.—Section through a pellet obtained from a low-dose leukemic cell culture after 7 weeks of tissue culture. A single particle is present between two cells. × 35,000

DISCUSSION

Dr. Salaman: Dr. Kirsten, do you feel you can distinguish between your model of malignant transformation, on the basis of those results, and the alternative model, namely, that there are two cells present from the start, one which supports multiplication and dies out in culture and the other which is transformed and is responsible for the neoplasia?

There is a curious parallel which seems to point to the latter model in the growth of Riley's lactic dehydrogenase-elevating virus in tissue culture. You can cultivate this agent in primary mouse embryo tissue cultures for a time and then these cells lose the virus, and if you take control cultures and keep them for the same length of time they become insusceptible to the virus.

On the other hand, if you grow the virus in peritoneal macrophages it will multiply as long as the cells survive. It appears, therefore, that some cell, possibly a macrophage, is dying out in the embryonic cultures, rendering the cultures less and less susceptible to infection. May not something of this kind be happening in your case?

Dr. Kirsten: I have no evidence it might be happening, but I cannot exclude it either. These are, of course, malignant cells from the very beginning. They were induced as thymic lymphomas *in vivo* and then transferred to the culture. We have attempted to transform normal cells into lymphoma or leukemic cells *in vitro*, but so far the results are not very encouraging.

Dr. Ioachim: We have now in our laboratory established cultures that originated from a thymic lymphoma primarily induced in a rat with Gross Passage A virus. These cultures, which were continuously grown for over 3 years, look exactly as they did in the beginning. One of them is a symbiotic culture in which the reticular cells grow attached to the glass and spread out, while the lymphocytes float in the supernatant. These cultures constantly release virus in abundance. The viral content was demonstrated on electron micrographs by Dr. L. Berwick and by bioassays in which up to 100% of inoculated animals died with leukemia in an average latency of 90 days. Our system is to assay these tissue cultures of rat origin in mice, which would prevent eventual grafting of cells. However, cell-free filtrates of cultures similarly produced 100% leukemia in susceptible animals.

In a second system we infected mice with the same Gross Passage A virus from normal embryonal rat thymus. These cultures grew abundantly and we have them now after 9 months *in vitro*. We did not rejuvenate them. They were trypsinized many times and there seems to be a difference between the control and virus-infected cultures.

These infected cultures also replicate the virus in high titer, as demonstrated by bioassays in susceptible strains of mice.

Antigenic Study on the Friend, Moloney and Rauscher Viruses With the Electron Microscope Agglutination Test [1, 2]

Sami A. Mayyasi, George Schidlovsky, Laurette M. Bulfone, *and* Nan L. Clifford, *The John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, New Jersey*

The agglutination of viruses by specific immune sera, as observed with the electron microscope, has been described for the avian erythroblastosis virus (*1*), poliovirus and bacteriophage (*2*), vaccinia virus (*3*), influenza virus (*4*, *5*), and human wart virus (*6*, *7*). Agglutination of the Rauscher murine leukemia virus by homotypic antibody was reported previously (*8*,*9*).

This report describes further study on the agglutination of Friend, Moloney, and Rauscher viruses by specific antisera. The agglutination technique was adapted to provide a serologic test to assay the agglutination potency of antisera, and also to detect antigenic cross-reaction among these viruses.

MATERIALS AND METHODS

Preparation of antisera.—Anti-Moloney virus serum was prepared in BDF_1 mice by the injection of Moloney virus derived from infected BALB/c mouse plasma. Diluted 1 : 10, 0.1 ml of the 10 times concentrate of the virus was injected intraperitoneally at 0, 2, and 4 weeks. The mice were bled 2 weeks later.

Anti-Moloney virus sera in rabbits were prepared by the intraperitoneal injection of 1 ml of 50 times concentrate of tissue culture virus derived from Manaker's MT-77 Moloney-virus-infected spleen cell line into rabbits (*10*). The virus, mixed with an equal volume of Freund's adjuvant, was injected on days 0 and 7. On day 21, 0.5 ml of the virus alone

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported by Public Health Service contract PH 43-62-144 from the National Cancer Institute.

was injected intramuscularly. The rabbits were bled 2 weeks after the last injection. Anti-Rauscher and anti-Friend viruses were prepared in C57BL mice by the same procedure described for the preparation of the anti-Moloney serum in mice.

Anti-Rauscher virus serum in rabbits was prepared by the injection of 10 times concentrate of Rauscher virus derived from BALB/c infected mice, and by the use of 50 times concentrate of virus derived from BALB/c mouse spleen-thymus cell line chronically infected with Rauscher virus (*11*). The immunization schedule was similar to that described for the Moloney virus.

Rabbit immune sera were absorbed with 0.1 ml of normal mouse plasma per 1 ml of immune sera to remove the antibodies formed against normal mouse plasma until no precipitin was detected upon repeating the absorption with normal mouse plasma.

Rabbit immune sera were absorbed 3 times with normal mouse spleen cells at the ratio of 1 × 10^8 cells per ml of immune serum. All sera were heat-inactivated at 56° C for 30 minutes before testing.

The agglutination test.—To perform the test, we mixed 0.2 ml of serial doubling dilutions of immune sera in 0.05 M sodium citrate with 0.2 ml of plasma or tissue culture virus. The mixture was incubated at room temperature for 30 minutes and overnight at +5° C. The contents were then transferred to 12 ml plastic Spinco centrifuge tubes. The tubes were filled completely with 0.05 M sodium citrate to dilute the serum protein, an excess of which interferes with the negative staining (*8*).

The tubes were centrifuged in the #40 rotor at 30,000 × *g* for 1 hour. The supernatants were removed by aspiration and the pellets were resuspended in 0.2 ml of 0.05 M sodium citrate. The virus was dispersed by pipetting 20 times with a 1 ml tuberculin syringe fitted with a 27-gauge needle. For negative staining, one part of the virus suspension was mixed with two parts of 2 percent potassium phosphotungstate, at *p*H 4.5, in a watch glass. The phosphotungstate was prepared by the use of 2 g of phosphotungstic acid dissolved in 100 cc of distilled deionized water and 5 N KOH added to adjust the *p*H. The sample was mixed thoroughly with the stain by pipetting. The film side of a carbon-coated grid was then applied to the surface of the virus stain mixture, immediately withdrawn on contact, drained into a filter paper, air dried, and observed in the electron microscope.

Serum neutralization test.—Equal volumes of 1:2 dilution of immune sera and 10 grams equivalent of plasma virus diluted 10^{-1}, 10^{-2}, and 10^{-3} were mixed. After the mixture was incubated at room temperature for 30 minutes and overnight at 5° C, 0.2 ml was injected intraperitoneally into BALB/c mice 4 to 5 weeks old; at least 10 mice were used per dilution. For the Moloney virus, newborn BALB/c mice 3 to 5 days old were used for the virus titration. All surviving mice were held for 120 days. At the end of the observation period, surviving mice were autopsied and organs were examined for evidence of virus infection.

RESULTS

Mixing the virus with homotypic antibody resulted in agglutination. This reaction resulted from the use of immune sera prepared in rabbits, mice, or monkeys, as with the Rauscher virus. Figure 1 illustrates the appearance of Rauscher virus when mixed with normal rabbit serum. The appearance of the same virus, when mixed with Rauscher virus immune serum prepared in rabbits, is shown in figure 2. Similar results were obtained with the Moloney and Friend viruses when combined with the homotypic antibody. No agglutination was observed with normal monkey, rabbit, or mouse sera.

Immune sera prepared against virus preparations derived from infected tissue culture cell lines or from infected mouse plasma showed the same agglutination. In addition, the intensity of the reaction was proportional to the dilution of the immune serum. A strong reaction showed large aggregates of the virus and the absence of free virus particles. In a weak reaction, the size of the aggregates was smaller and free virus particles were present.

Optimum conditions for performing the test depend upon the availability of clean and fresh virus preparations. Virus preparations containing a count of 100 to 500 particles per square (200 mesh) yield the most reproducible results.

The agglutination capacity of the immune sera was removed by absorption with the virus pellet. The reaction was not diminished when the antisera were absorbed with normal mouse cells. Antisera prepared against unrelated viruses were also tested. Immune sera prepared against type II poliovirus, PR-8 strain of influenza virus, SV40, or Rous sarcoma virus failed to agglutinate Rauscher virus. Furthermore, vaccinia virus was not agglutinated by Rauscher virus immune serum.

The agglutination capacity of the immune sera was related to its neutralizing capacity. The agglutination titers of some Rauscher virus immune sera were 1:2, 1:20, and 1:60 with corresponding neutralizing indexes of $10^{1.5}$, $10^{2.7}$, and $>10^3$, respectively.

Antigenic cross-reaction between the three leukemic viruses was investigated by the agglutination test. Antisera with a strong neutralizing activity prepared against Friend, Moloney, and Rauscher viruses were used to detect cross-reaction between these viruses by the heterotypic antibody. Table 1 demonstrates the homotypic antibody to yield the highest agglutination titer. These results indicate that the Friend, Moloney, and Rauscher viruses shared a common antigenic component. Figure 3 illustrates the appearance of the Moloney virus when combined with Rauscher virus immune serum. The appearance of the Rauscher virus, when mixed with Friend immune serum, is shown in figure 4. Anti-Moloney virus immune serum neutralized the infectivity of Rauscher virus when measured by the serum neutralization test (table 2). The neutralization of Moloney virus by the Rauscher virus immune serum is shown in table 3.

TABLE 1.—Agglutination of murine leukemia viruses by homotypic and heterotypic antibodies

Immune serum	Species	Agglutination titer*		
		Rauscher virus	Moloney virus	Friend virus
Rauscher virus immune serum	Rabbit	128	16	64
	C57BL mice	16	4	4
	Rabbit	160	32	64
Moloney virus immune serum	Rabbit	8	64	8
	BDF_1 mice	4	16	2
	Rabbit	8	16	8
Friend virus immune serum	C57BL mice	16	4	32
	C57BL mice	4	4	8

*Highest dilution of immune serum that agglutinates the virus.

TABLE 2.—Neutralization of Rauscher and Moloney viruses with Moloney virus immune serum

	Dilution of Rauscher virus*			Dilution of Moloney virus†		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Moloney virus immune serum‡	7/10§	0/10	0/9	0/10	1/11	0/10
Normal rabbit serum	5/6	6/7	2/10	12/12	8/11	6/10

*Tenfold concentration of Rauscher mouse plasma virus.

†Tenfold concentration of Moloney mouse plasma virus.

‡Immune serum prepared in rabbits against purified Moloney virus cultivated from Manaker's MT-77 cell line.

§Number of mice infected with leukemia
Total number of mice inoculated

TABLE 3.—Neutralization of Rauscher and Moloney viruses by Rauscher virus immune serum

	Dilution of Moloney virus*			Dilution of Rauscher virus†		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Rauscher virus immune serum‡	5/15§	0/13	0/13	0/8	0/10	0/10
Normal mouse serum	9/10	9/10	3/16	10/10	10/10	3/9

*Tenfold concentration of Moloney plasma virus.

†Tenfold concentration of Rauscher plasma virus.

‡Immune serum prepared in BDF_1 mice against purified Rauscher mouse plasma virus.

§Number of mice infected with leukemia
Total number of mice inoculated

In both cases, neutralization of the virus by the homotypic antibody was stronger than the reaction with the heterotypic antibody.

DISCUSSION

Agglutination of mouse leukemia viruses by specific immune serum was shown with the Friend, Moloney, and Rauscher viruses. The specificity of the reaction was verified by the use of immune sera unrelated to this group of viruses. The absence of agglutination of vaccinia virus with Rauscher immune serum supported the above-mentioned data. The antibody responsible for agglutination of the virus was associated with the virus particles rather than with soluble antigens. The agglutination activity of the immune sera was removed by absorption of the antibody by pellets of the virus.

The cross-reaction observed between the Moloney and Rauscher viruses by the serum neutralization tests, and the cross-agglutination between the Friend, Moloney, and Rauscher viruses suggest the presence of a common group antigenic component. Other workers have demonstrated a common antigenic component among the three viruses by the cytotoxicity test (*12*, *13*) and by the complement fixation test (*14*).

The antigenic relationship of the Friend, Moloney, and Rauscher viruses to other members of the murine leukemia virus group is being studied. This agglutination test provides a serologic technique to detect the antigenic relationship between known and newly isolated oncogenic viruses.

SUMMARY

Friend, Moloney, and Rauscher mouse leukemia viruses were agglutinated by antisera prepared in rabbits and mice. A serologic test was described to titrate the agglutination capacity of antisera and to study the antigenic relationship between the three viruses. A common antigenic component was detected in these viruses by the agglutination test. A cross-reaction between the Moloney and Rauscher viruses was demonstrated by the serum neutralization test.

REFERENCES

(*1*) Bonar, R. A., Sharp, D. A., Beard, D., and Beard, J. W.: Identification of avian erythroblastosis virus by precipitation with chicken immune serum. Proc Soc Exp Biol Med 92: 774–778, 1956.

(*2*) Anderson, T. F., Yamamoto, N., and Hummeler, K.: Specific agglutination of phages and poliovirus by antibody molecules seen in the electron microscope. J Appl Physics 32: 1693, 1961.

(*3*) Bayer, M. E.: Electron optical studies on immunological reactions of vaccinia virus particles in suspensions and in broken-up cells. Fifth Int Cong Electron Microscope, X–2 (Breese, S. S., ed.). New York, Academic Press Inc., 1962.

(*4*) Bayer, M. E., and Mannweiler, E.: Antigen-antibody reactions of influenza virus as seen in the electron microscope. Arch Ges Virusforsch 13: 541–547, 1963.

(*5*) Laferty, J. J., and Oertelis, S.: The interaction between virus and antibody. III. Examination of virus-antibody complexes with the electron microscope. Virology 21: 91–99, 1963.

(*6*) ALMEIDA, J., CINADER, B., and HOWATSON, A.: The structure of antigen antibody complexes. A study by electron microscopy. J Exp Med 118: 327–340, 1963.

(*7*) OROSZLAN, S. I., and RICH, M. A.: Human wart virus. Science 146: 531–533, 1964.

(*8*) SCHIDLOVSKY, G., and MAYYASI, S. A.: Factors affecting the morphology of murine leukemia viruses. Proc Amer Assoc Cancer Res 6: 56, 1965.

(*9*) MAYYASI, S. A., SCHIDLOVSKY, G., and BULFONE, L. M.: Agglutination of murine leukemia viruses by specific immune sera as observed by the electron microscope. Virology 27: 431–434, 1965.

(*10*) MANAKER, R. A., STROTHER, P. C., MILLER, A. A., and PICZAK, C. V.: Behavior *in vitro* of a mouse lymphoid-leukemia virus. J Nat Cancer Inst 25: 1411–1419, 1960.

(*11*) WRIGHT, B. S., and LASFARGUES, J. C.: Long-term propagation of the Rauscher murine leukemia virus in tissue culture. J Nat Cancer Inst 35: 319–327, 1965.

(*12*) KLEIN, E., and KLEIN, G.: Antigenic properties of lymphomas induced by the Moloney agent. J Nat Cancer Inst 32: 547–568, 1964.

(*13*) BOYSE, E. A., OLD, L. J., and LUELL, S.: Mouse leukaemias. Typing of mouse leukaemias by serological methods. Nature (London) 201: 777–779, 1964.

(*14*) HARTLEY, J. W., ROWE, W. P., CAPPS, W. I., and HUEBNER, R. J.: Complement fixation and tissue culture assays for mouse leukemia viruses. Proc Nat Acad Sci USA 53: 931–938, 1965.

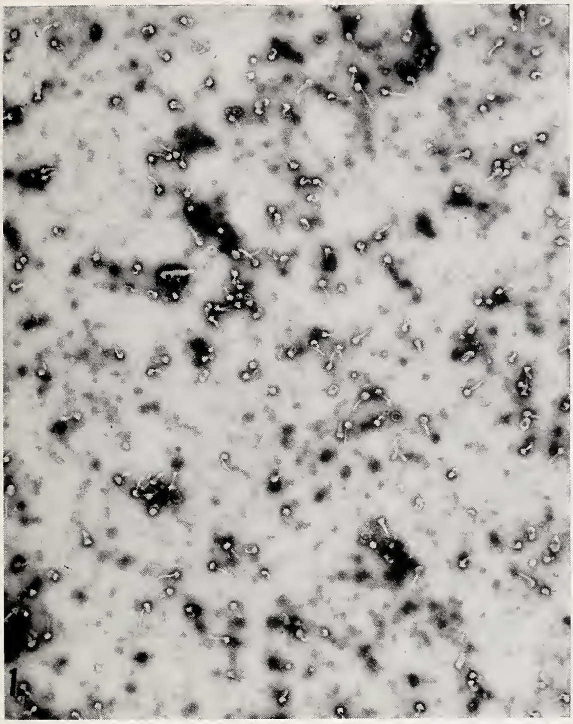


Figure 1.—Plasma Rauscher virus mixed with normal rabbit serum. × 11,000

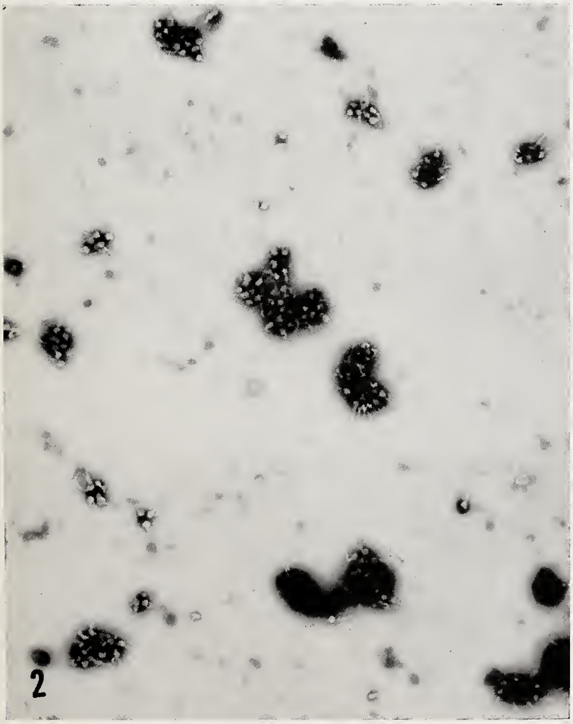


Figure 2.—Plasma Rauscher virus mixed with immune serum prepared in rabbits against tissue culture Rauscher virus. × 11,000

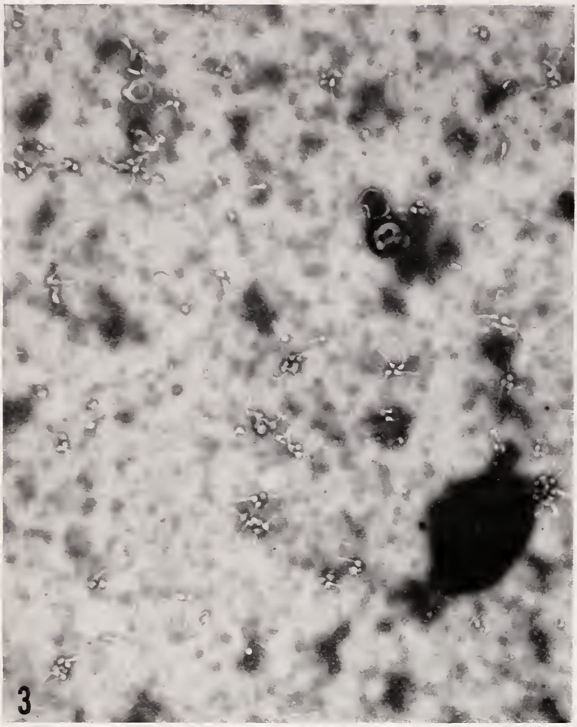


FIGURE 3.—Tissue-culture Moloney virus mixed with immune serum prepared in mice against mouse plasma Rauscher virus. × 11,000

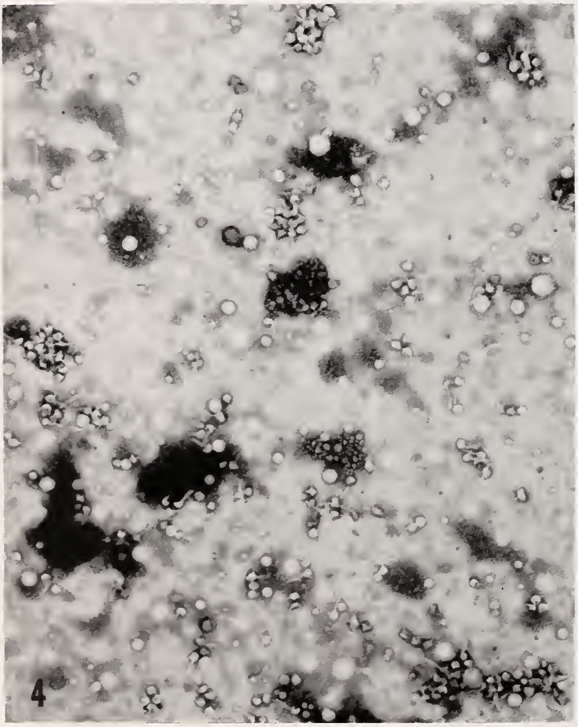


FIGURE 4.—Plasma Rauscher virus mixed with immune serum prepared in mice against mouse plasma Friend virus. × 11,000

DISCUSSION

Dr. Sinkovics: Dr. Mayyasi. If you expose these viruses with immune serum and complement, would they dissolve?

Dr. Mayyasi: We have not tried this, but all our sera were heat-inactivated before they were tested.

Dr. Tennant: I think it would be an interesting corollary to your study, to check for agglutination of these viruses by the antisera now available at The Jackson Laboratory, which are specific to various of the mouse isoantigens.

Dr. Mayyasi: This is a good suggestion. We have tried to use this test to differentiate between some latent viruses, and we have a virus that was isolated by Dr. Wright from a normal mouse spleen thymus cell line (JLS-V6). This virus was shown to be unrelated to the Rauscher virus. Immune serum prepared against this latent virus failed to agglutinate the Rauscher virus. On the other hand, it agglutinates its own virus. So we feel there is quite a specificity in this reaction.

Enhancing Effect of Chemical Carcinogens on Experimental Viral Infection: Its Significance and Probable Mechanism[1]

MARIA L. DURAN-REYNALS, *Department of Pathology, Albert Einstein College of Medicine, New York, New York*

THIS subject has been reviewed lately, in order of publication, by us (*1*), by Salaman and Roe (*2*), and by Martin (*3*). The data discussed in these reviews concern the enhancing effect of chemical carcinogens on the susceptibility of the host to experimental infection by either "non-neoplastic" or "neoplastic" viruses. Briefly summarized, these data indicate the following.

Under certain conditions, the joint administration of a chemical carcinogen and a non-neoplastic virus enhances the acute response, which is rapidly followed by the death of the host. This was first demonstrated by Teague and Goodpasture (*4*) with the virus of herpes simplex. However, under other conditions, the animals survive the initial acute effects of the virus but their neoplastic response is subsequently considerably enhanced; this was first demonstrated by F. Duran-Reynals (*5*) with vaccinia dermal infection.

The joint administration of a chemical carcinogen and a neoplastic virus results consistently in an enhancement of the neoplastic properties of the virus, as was first demonstrated by Rous and Kidd (*6*) with the papilloma virus. This enhancing effect on the properties of the virus manifests itself variously by an acceleration of the neoplastic process and/or an increase in the incidence of tumors, as well as in the induction of apparently malignant tumors by viruses which ordinarily induce benign lesions; this was first demonstrated by Ahlström and Andrewes (*7*) with the fibroma virus.

The problem of leukemia has been, to our knowledge, the one least studied so far in relation to the effects of chemical carcinogens on viral infection, except for the following findings.

Chieco-Bianchi *et al.* (*8*) reported that C57BL mice, inoculated intraperitoneally first with the Graffi leukemia virus and 4 days later with urethan, developed a much higher incidence of leukemia (55%) than mice inoculated with the virus alone (5%) or mice inoculated with urethan alone (8%). Urethan had no effect on the response to the virus of C3H mice which appeared to be resistant to this virus.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

Findings reported by us (*1*) indicate that in mice rendered susceptible to vaccinia virus by pretreatment with cortisone, vaccinia dermal infection increased significantly the incidence of leukemia in the presence of a chemical carcinogen. Whether the virus was directly responsible for the leukemogenic effect is not known. Under certain conditions, the incidence of leukemia was directly related to the severity of the virus-induced skin lesion rather than to the dose of the chemical carcinogen. We also note that vaccinia infection has been associated with the development of leukemia in mice infected with this virus shortly after birth (*9*), as well as in humans following smallpox vaccination (*10–12*).

The mechanism responsible for the enhancing effect of chemical carcinogens on experimental viral infection has not been determined. There are, however, data which give some indication as to the possible nature of this mechanism. These data concern the effects of chemical carcinogens on viruses other than the leukemogenic ones, about which there is by comparison very little information in this respect, as already mentioned. For this reason, we will discuss these findings at this Conference since they seem to suggest that the same mechanism may be responsible for the neoplastic response in a variety of systems. These findings, which in part we have already discussed (*1*), indicate the following: Chemical carcinogens do not seem to modify the properties of a virus introduced experimentally into the host but rather induce changes in the host which result, in the presence of the virus, in an enhancement of the neoplastic response. Such changes appear to be induced also by other agents, such as X rays and cortisone, as well as by factors endogenous to the host, such as age. These agents as well as chemical carcinogens have in common the property of inhibiting the immunological processes, which also vary considerably in their level of activity depending on the age of the host. In certain systems, the enhancing effect of chemical carcinogens on the neoplastic response to viral infection lasts a very short time and cannot, therefore, be detected unless infection with the virus is initiated during this critical period.

APPARENT NONOCCURRENCE OF VIRUS VARIATION IN THE CARCINOGEN-TREATED ANIMAL

Under certain conditions, the same virus may induce in the normal and in the carcinogen-treated animal totally different effects. Herpes simplex virus, virtually nonpathogenic for normal rabbit skin, induced in tarred rabbit skin generalized, severe lesions eventually causing death of the host (*4*). Furthermore, these lesions were clinically indistinguishable from those induced by herpes zoster infection in the normal host. Similar findings were also obtained with the fibroma virus (*7*), which mimicked in the carcinogen-treated rabbit the clinical effects ordinarily induced by the myxoma virus in the normal rabbit. However, we wish to emphasize that neither of these two viruses, nor other viruses tested (*1*), seemed to change in their properties for the normal host after passage through the carcinogen-treated animal. It appears, therefore, that the often atypical behavior of the virus in the presence of the carcinogen is the result of changes apparently induced in the host by the chemical. With this in mind, we will now indicate the following pertinent findings.

CHEMICAL CARCINOGENS AND IMMUNOLOGICAL RESPONSE

Considerable importance has been attached lately to findings, recently reviewed by Berenbaum (*13*), indicating that chemical carcinogens inhibit the immunological processes.

It has been observed (*13*), however, that inhibition of the immunological response by chemical carcinogens is not followed in all systems by the development of tumors. This is not surprising in view of findings obtained in experiments dealing with the combined effects of carcinogenic agents and viral infection. Under certain conditions, the animals treated with both the chemical and the virus develop a much higher incidence of tumors than the animals treated only with the chemical and, in some systems, the animals receiving the chemical alone develop no tumors. We reported (*1*) that vaccinia dermal infection in mice treated with cortisone and a chemical carcinogen was followed by a significantly higher incidence of neoplasms than in the control mice, which received the carcinogen but no virus, and by the development of tumors at the precise site of viral infection. The mice used for these studies were randombred albinos. We have repeated the experiment in DBA/2 inbred mice. The incidence in these mice of papillomas at the site of vaccinia dermal infection is at present 10 to 15%, which is highly significant because none of the DBA/2 mice from the various control groups has developed a single skin tumor thus far. These findings suggest that, in certain systems, other factors besides the carcinogen are needed for the neoplastic response to take place. Whether the carcinogen impairs the immunological response in these systems has not been determined; it would be of much interest to study this.

CHEMICAL CARCINOGENS AND INTERFERON

De Maeyer *et al.* (*14–16*) have recently published a series of papers which indicate that chemical carcinogens inhibit the synthesis of interferon *in vitro*. This is of considerable interest in reference to the fact that carcinogen-treated animals develop, under certain conditions, tumors only at the precise site of application of the chemical. Furthermore, it is of interest to note in this respect the findings observed by others (*17*) and by us (*1*) in animals that received either skin paintings of a chemical carcinogen or were also inoculated in the painted skin with a virus. In the animals that received only the chemical, tumors developed at random at different sites on the painted skin. In the animals receiving both the chemical and the virus, tumors developed preferentially (or almost exclusively) at the precise site of virus inoculation in the painted skin.

CHEMICAL CARCINOGENS AND AGE OF THE HOST

Papilloma virus infection of carcinogen-treated adult rabbits was followed almost immediately by the appearance of carcinomas at the infected site (*6*). It is, however, well known that infection of normal rabbit skin with this virus is also followed frequently by the appearance of carcinomas at the infected site several months later. It thus seems that, under these conditions, the carcinogen simply accelerates a process that is apt to take place also in the untreated, aging animal. One might then say that, in this case, the carcinogen mimics the effects of increasing age.

CHEMICAL CARCINOGENS, X RAYS, CORTISONE, AND AGE OF THE HOST

I. In mice naturally infected with the polyoma virus, this virus was activated from the latent state into the tumor-inducing state by X rays, as reported by Gross (*18*), and by cortisone, as reported by Woolley and Small (*19*). These findings indicate that both the X rays and the cortisone had created in these animals the conditions brought about by age, if the mice are infected with the virus experimentally at birth. Subsequently, Rawson *et al.* (*20*) reported that, in mice infected at birth with the polyoma virus and treated later on with chemical carcinogens, the activation of the virus from the latent state into the tumor-inducing state was accelerated and the incidence of polyoma-induced tumors was increased. These findings suggest that X rays, cortisone, and chemical carcinogens can have similar effects in that the three can mimic respectively, in the same system, the effects of increasing age. In this case, therefore, the carcinogen mimicked the effects of age in the polyoma-infected mice as it mimics the effects of age in the papilloma-infected rabbit and we note, in this respect, that both the polyoma and papilloma viruses belong to the same papova family of viruses.

II. Findings which in a way confirm the above observations were obtained also with another virus, not of the papova group, but of the pox group, namely, the Shope fibroma virus of the rabbit. It is well known that this virus induces in the normal domestic rabbit skin fibromas which regress in a few weeks leaving the host immune to the virus apparently for life. However, this virus induced generalized, invasive, progressively growing tumors in the domestic rabbit treated with: 1) chemical carcinogens, as reported by Ahlström and Andrewes (*7*); 2) X rays, as reported by Clemmesen (*21*); and 3) cortisone, as reported by Harel and Constantin (*22*). The fibroma virus also induced this type of tumor in *untreated newborn* rabbits, as reported by F. Duran-Reynals (*23*), who demonstrated that these animals are incapable of producing antibodies against the virus (*24*). These findings indicate that, in this system, X rays, cortisone, and chemical carcinogens appeared to reverse the effects of age. Thus, the normally highly resistant adult rabbit responded to the virus, in the presence of each one of these three agents, as the highly susceptible, immunologically defenseless newborn rabbit.

TRANSITORY EFFECTS OF CHEMICAL CARCINOGENS

We have indicated above the similarities between the effects of chemical carcinogens and the effects of aging, both in the polyoma-infected mouse and in the papilloma-infected rabbit. In the latter, however, there seems to be a basic difference in certain respects between the effects of the carcinogen and those of age. The benign tumors originally induced by the papilloma virus in the rabbit may become malignant as the host ages, but we do not know that infection with this virus in older rabbits is ever followed by the immediate appearance of malignant tumors. Yet this is what happens when infection with this virus is induced in the presence of the carcinogen (*6*). It is, however, possible that theoretically this difference may be more apparent than real, in view of the following observations. When the rabbits received the carcinogen *before* the virus, the neoplastic effects of the latter were enhanced *only* if infection was initiated very soon after administration of the chemical. As reported by Friedewald (*25*), methylcholanthrene paintings enhanced the susceptibility of rabbit skin to the neoplastic effects of the papilloma virus, but only for about 2 weeks after treatment with the carcinogen was discontinued. After these 2 weeks the carcinogen-treated skin responded to the virus the same as the normal skin. In view of these findings Friedewald (*25*) pointed out that ". . . the increased reactivity (to the papilloma virus) of methylcholanthrene-treated skin is transitory and is lost 2–4 weeks after the treatments are stopped."

From the above it could be postulated that the aging process induces, perhaps repeatedly, a transitory enhancement of the susceptibility to viral infection which, by virtue of its short duration, benefits only viruses already present in the host.

Transitory effects of chemical carcinogens similar to those reported by Friedewald have been observed recently by Tanaka and Southam (*17*) in mice painted with methylcholanthrene and inoculated in the painted skin with a supposedly non-neoplastic virus, namely, herpes simplex. Inoculation of this virus was followed in these animals by an enhancement of the neoplastic response as well as by the development of papillomas at the site of virus inoculation. However, neither of these effects was observed if the virus was inoculated 14 days after the first application of the chemical, that is, 9 days after treatment was discontinued. Tanaka and Southam (*17*) postulated that ". . . the increase in papilloma incidence occurred when herpes virus propagation coincided in time and place with the epidermal hyperplasia caused by the chemical. . . ."

Results have also been observed which suggest that certain effects of chemical carcinogens might come to an end even before treatment is discontinued. Tanaka and Southam (*26*) have reported that West Nile virus, inoculated intraperitoneally into mice that received 10 paintings of methylcholanthrene in 10 days, enhanced the neoplastic response of these animals. This effect, however, took place when the virus was inoculated after the third painting but not if it was inoculated after the tenth painting.

The transitory effects of chemical carcinogens summarized above suggests that the chemical had modified locally the response of the susceptible cell to the virus by inducing directly cellular changes which apparently were not transmitted indefinitely to succeeding cell generations. One possible indication as to the nature of these changes may be the inhibiting effect of chemical carcinogens on interferon synthesis reported, as mentioned above, by De Maeyer *et al.* (*14–16*).

Another indication as to the possible mechanism of these transitory effects of chemical carcinogens on viral infection may be found in the following observations.

In the experiments summarized above, concerning the effects of the fibroma virus in rabbits treated, respectively, with cortisone, chemical carcinogens, or X rays, it should be stressed that only in the latter system was the immunological response to the virus studied in some detail (*21*). These studies revealed that the X-ray-treated rabbits became immune to the fibroma virus 8 to 14 days after primary infection, whereas the normal control rabbits became immune 3 to 5 days after primary infection. The X rays, therefore, had suppressed the immune response to the virus for a few days only. However, the virus inoculated into the X-ray-treated rabbits during these critical days induced generalized, invasive tumors which grew until the death of the host, *i.e.*, the same type of tumors as in the carcinogen-treated rabbits.

CONCLUSIONS

From the findings summarized in this paper, the following conclusions may be reached to construct at least a working hypothesis.

1) The enhancing effects of chemical carcinogens on the neoplastic response to experimental viral infection may be mediated by an impairment of the natural defenses against the virus probably at both the systemic and cellular levels.

2) This impairing effect need not be quantitatively highly significant and persists probably for a very short time.

3) The mechanism by which this impairing effect of chemical carcinogens enhances the neoplastic response may be essentially the same as that by which agents, such as X ray and cortisone, as well as factors pertaining to the host, such as age, induce similar effects.

It might not be easy to verify experimentally the validity of these conclusions, as such conclusions postulate a cause-effect relationship between probably minor, ephemeral, nonspecific effects and the eventual development of malignancy. The attempt to demonstrate such a relationship might not be very rewarding, therefore, unless the right system is found—perhaps by means of studies similar to those discussed in this paper.

REFERENCES

(*1*) Duran-Reynals, M. L.: Combined effects of chemical carcinogenic agents and viruses. Progr Exp Tumor Res 3: 148–185, 1963.

(*2*) Salaman, M. H., and Roe, F. J. C.: Cocarcinogenesis. Brit Med Bull 20: 139–144, 1964.

(*3*) Martin, C. M.: Viruses-carcinogens interactions. Bact Rev 28: 480–489, 1964.

(*4*) Teague, O., and Goodpasture, E.: Experimental herpes zoster. J Med Res 24: 185–200, 1923.

(*5*) Duran-Reynals, F.: Studies on the combined effects of chemical carcinogens, hormones and virus infection. Texas Rep Biol Med 15: 306–333, 1957.

(*6*) Rous, P., and Kidd, J. G.: The carcinogenic effect of a papilloma virus on carcinogenic hydrocarbons on interferon synthesis and virus plaque formation. J Nat Cancer Inst 34: 265–276, 1965.

(*7*) Ahlström, C. G., and Andrewes, C. N.: Fibroma virus infection in tarred rabbits. J Path Bact 47: 65–86, 1938.

(*8*) Chieco-Bianchi, L., Fiore-Donati, L., De Benedictis, G., and Tridenti, G.: Influence of urethane on susceptibility to leukemia induced by Graffi virus in adult mice. Nature (London) 199: 292–293, 1963.

(*9*) Mazurenko, N. P.: Induction of leukemia in mice with infectious viruses and the significance of the latter in the etiology of the disease. Probl Onkol 6: 873–882, 1960.

(*10*) Lien-Keng, K.: A hyperacute case of erythroleukemia treated with aminopterin. Acta Paediat (Stockholm) 42: 157–165, 1953.

(*11*) Helman, J.: Vaccinia as a possible carcinogen. Lancet: p. 50, 1957.

(*12*) Andersen, H. J., Nilsson, L. R., and Lund, E.: Acute leukemia following smallpox vaccination in a ten-month-old infant. Acta Paediat (Stockholm) 54: 383–386, 1965.

(*13*) Berenbaum, M. C.: Effects of carcinogens on immune processes. Brit Med Bull 20: 159–164, 1964.

(*14*) De Maeyer, E., and De Maeyer-Guignard, J.: Inhibition by 20 methylcholanthrene of interferon production in rat cells. Virology 20: 536–539, 1963.

(*15*) ———: Inhibition by 3-methylcholanthrene of interferon formation in rat-embryo cells infected with Sindbis virus. J Nat Cancer Inst 32: 1317–1331, 1964.

(*16*) De Maeyer-Guignard, J., and De Maeyer, E.: Effect of carcinogenic and non-carcinogenic hydrocarbons on interferon synthesis and virus plaque formation. J Nat Cancer Inst 34: 265–276, 1965.

(*17*) Tanaka, S., and Southam, C. M.: Joint action of herpes simplex virus and 3-methylcholanthrene in production of papillomas in mice. J Nat Cancer Inst 34: 441–451, 1965.

(*18*) Gross, L.: Oncogenic Viruses. New York, Pergamon Press, 1961, 298 pp.

(*19*) Woolley, G. W., and Small, M. C.: Strain specificity of leukemia agent. Ann NY Acad Sci 68: 533–539, 1957.

(*20*) Rawson, K. E. K., Roe, F. J. C., Ball, J. K., and Salaman, M. H.: Induction of tumors by polyoma virus; enhancement by chemical agents. Nature (London) 191: 893–985, 1961.

(*21*) Clemmesen, J.: The influence of roentgen radiation on immunity to Shope fibroma virus. Amer J Cancer 35: 378–385, 1939.

(*22*) Harel, J., and Constantin, T.: Sur la malignité des tumeurs provoquées par le virus fibromateux de Shope, chez le lapin nouveau-né et le lapin adulte traité par des doses massives de cortisone. Bull Assoc Franc Cancer 41: 483–499, 1954.

(*23*) Duran-Reynals, F.: Production of degenerative inflammatory or neoplastic effects in the newborn rabbit by the Shope fibroma virus. Yale J Biol Med 13: 99–110, 1940.

(*24*) ———: Immunological factors that influence the neoplastic effects of the rabbit fibroma virus. Cancer Res 5: 25–39, 1945.

(*25*) Friedewald, W. F.: Cell states as affecting susceptibility to a virus; enhanced effectiveness of rabbit papilloma virus on hyperplastic epidermis. J Exp Med 75: 197–220, 1942.

(*26*) Tanaka, S., and Southam, C. M.: Joint action of West Nile virus and chemical carcinogens in production of papillomas in mice. J Nat Cancer Inst 29: 711–722, 1962.

Induction of a Leukemogenic Agent by a Chemical Carcinogen in Inbred Mice[1]

L. A. Zilber *and* Z. A. Postnikova, *Gamaleya Institute, Moscow, USSR*

THE problem of the role of viruses in carcinogenesis, induced by different chemical and physical factors, is studied by many investigators. It was repeatedly reported that viruses were isolated from tissues affected by neoplastic processes induced by chemical (*1–3*) and physical (*4–6*) factors.

Oncogenic action of cell-free extracts from tumors induced by 7,12-dimethylbenz[*a*]anthracene in mice of low-tumor strains CC57W, CC57BR, and C57BL/10ScSn was studied in the present work.

MATERIALS AND METHODS

Carcinogen was injected subcutaneously in doses of 50 to 100 μg in olive oil into 2- to 6-day-old mice. Beginning at the age of 2 months, mice were examined twice weekly.

Mice treated with the carcinogen developed sarcomas, papillomas, and cancer of the skin, as well as leukemias. All of these tumor tissues were used for preparation of cell-free extracts injected into newborn mice. Only very young tumors detected within 5 to 15 days before an experiment were used for preparation of extracts. Those from sarcomas were usually prepared from a pool of tumors of 2 to 4 mice, extracts from papillomas of 15 to 20 mice, and extracts of leukemic tissues from organs of each mouse.

Tumor tissues were triturated in Hank's solution, 1:3. Then the homogenates were centrifuged at 2,500 and 10,000 rpm for 20 and 30 minutes. The supernatant was centrifuged once more at 2,500 rpm for 15 minutes, and the bottom layer was tested for possible presence of cells. In no instance were cells revealed. Some extracts were also filtrated through Berkefeld's V candle.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

A dose of 0.05 to 0.1 ml of the supernatants or filtrates was injected subcutaneously or intraperitoneally into mice 24 to 48 hours after birth.

RESULTS

Table 1 presents results obtained when these extracts were injected into newborn mice of different strains that survived for 9 months after treatment. As seen from this table, mice receiving cell-free extracts of sarcomas induced by the carcinogen developed leukemias; the incidence varied in different strains. Thus, 8 of 73 CC57W mice, 2 of 43 CC57BR mice, and 1 of 59 C57BL/10ScSn mice developed leukemias.

Similar results were also observed in mice that received extracts from leukemic tissues of animals treated with carcinogen. Thus, 5 of 21 CC57W mice treated as mentioned above, 3 of 19 C57BL/10ScSn mice, and none of 6 CC57BR mice developed leukemias.

Some extracts were tested in all three mouse strains, others in one or two. Analysis of the described experiments demonstrated that the leukemogenic agent was in different mouse strains in 3 of 22 tested sarcoma extracts and in 4 of 7 extracts of leukemic lesions. The leukemogenic agent was not detected in C57BL/10ScSn mice with carcinogen-induced sarcomas.

Table 2 shows experiments in which leukemias appeared after injection of extracts from sarcomas or from organs of leukemic mice. The first 3

TABLE 1.—Neoplasms in mice treated with extracts of carcinogen-induced tumors

Number of experiments	Tumors of donors	Mouse strains of recipients	Number of mice: Survived for 9 months	Number of mice: With leukemias	Number of mice: With tumors of other localizations
15	Sarcomas	CC57W	73	8	3
5	Leukemias	CC57W	21	5	1
1	Cancer of skin	CC57W	3	0	0
1	Papilloma of skin	CC57W	3	0	0
2	Heated extracts of sarcomas	CC57W	17	0	0
	Total		117	13	4
8	Sarcomas	CC57BR	43	2	3
3	Leukemias	CC57BR	6	0	0
4	Papillomas of skin	CC57BR	37	0	1
3	Heated extracts	CC57BR	23	0	0
	Total		109	2	4
9	Sarcomas	C57BL/10ScSn	59	1	1
4	Leukemias	C57BL/10ScSn	19	3	1
2	Papillomas of skin	C57BL/10ScSn	20	1	0
2	Heated extracts	C57BL/10ScSn	13	0	0
	Total		111	5	2

TABLE 2.—Leukemias in mice treated with cell-free extracts of carcinogen-induced tumors*

	Cell-free extracts injected into mice at birth and prepared from:		Recipients					
			CC57W		CC57BR		C57BL/10ScSn	
Serial No. of experiments	Strains of donors	Tumors induced by carcinogen	Survived for more than 9 months	With leukemias	Survived for more than 9 months	With leukemias	Survived for more than 9 months	With leukemias
21	CC57BR	Sarcomas of 2 mice			4	1(16)		
27	CC57BR and CC57W	Sarcomas of 4 mice	7	4 (13–20)	4	1(13)	2	1(18)
34	CC57BR	Sarcomas of mice	9	4 (11–22)	10	0		
22	CC57BR	Leukemia	8	3 (15–18)	1	0	9	0
29	CC57W	Leukemia	4	1 (16)	10	0		
45	CC57W	Leukemia					6	3 (9. 5–13)
48	CC57BR	Leukemia	4	1 (24)				
Total			32	13	29	2	17	4
% leukemias				40. 6		7		23. 5

*Time in months when leukemias were found is indicated in parentheses.

lines demonstrate the incidence of leukemias after injection of extracts from sarcomas of polymorphocellular or spindle-cellular structure.

The subsequent 4 lines present the data of experiments in which organs of mice (spleen, liver, thymus, and lymph nodes) with leukemias induced by carcinogen were used for extract preparation. All these leukemias were slightly differentiated hemocytoblastosis or hemocytoblastosis-myelosis.

In summary, experiments presented in table 2 indicate leukosis arose in 13 of 32 CC57W mice (40.6%), in 2 of 29 CC57BR mice (7%), and in 4 of 17 C57BL/10ScSn mice (23.5%). The 45th experiment showed that of 6 C57BL/10ScSn mice, 3 developed leukemias, and in addition, one female killed at 16 months had an adenocarcinoma of the mammary gland.

Generally, autopsy of mice with leukosis revealed a pronounced enlargement of the spleen. The liver and retroperitoneal, mesenteric, and submaxillary lymphatic nodes were increased in size and there was a slight enlargement of inguinal and axillary nodes. The thymus was usually enlarged (fig. 1).

Histological and cytological examinations showed pronounced characteristic infiltration of the liver, spleen, kidneys, lungs, thymus, and lymph nodes (figs. 2, 3, and 4).

The leukemias might be a slightly differentiated form of the hemocytoblastic type; sometimes cellular elements tend to differentiate toward the myeloid type.

For a study of the nature of these leukemias, cell-free extracts were prepared, as described above, from a supernatant obtained from a homogenate of leukemic organs, spleen, lymph nodes, and thymus. Cell-free extracts were injected into newborn mice as described below.

A filtrate of organs of a leukemic CC57W mouse was prepared through Berkefeld's V candle. This mouse developed leukemia 4.5 months after treatment with the carcinogen. The filtrate was injected into 3-day-old CC57W animals.

One of 4 inoculated mice developed leukemias within 16 months. Homogenates were prepared from leukemic tissues of this animal. The cell-free supernatant was inoculated into newborn mice of the same strain. Leukemias arose in 3 of 5 mice in 9, 12.5, and 17 months. The same supernatant, when heated at 80° C for 30 minutes, caused no disease. The subsequent passage with a cell-free material gave the following results: 7 of 12 inoculated CC57W mice became ill within 4.5 to 10.5 months; in all 5 inoculated C57BL/10ScSn mice the disease was demonstrated. A heated extract was inactive.

At present, 6 of 13 CC57W mice of the fifth passage are ill. The disease appeared within 3.5 to 7.5 months. Leukemias were of the same type as primary ones.

Other leukemic strains were isolated by the same method. They are under study in our laboratory now.

Passages were also successful with cellular material.

At present, we are studying the relation of isolated leukemic agent to other leukemic viruses.

DISCUSSION

Our data show that, in young tumors and leukemic tissues induced in low-leukemia newborn mice by injection of a carcinogenic substance, there might be a leukemogenic agent which is apparently of viral origin. This agent retains its leukemogenic activity while passaged in mice.

The question arises as to whether this agent originates in carcinogenesis induced by a carcinogenic substance. For the present, we have no data to answer this question. The carcinogenic agent possibly creates conditions for activation of a latent leukemic virus already present in mice. Perhaps a carcinogenic substance derepresses genetic information brought by the virus in a cell and already integrated with it. Too, the virus might not play any part in a like carcinogenesis and settles in a carcinogen-induced tumor.

All these possibilites need experimental study.

REFERENCES

(*1*) Zilber, L. A.: On the filtrability of tumors induced by 1,2,5,6 dibenzanthracene. Amer Rev Soviet Med 4: 100–105, 1946.

(*2*) Ageenko, A. I.: Filtrable virus-like agent isolated from tumours induced by carcinogenic substances. Folia Biol (Praha) 8: 7–11, 1962.

(*3*) Toth, B.: Development of malignant lymphomas by cell-free filtrates prepared from chemically induced mouse lymphomas. Pro Soc Exp Biol Med 112: 873–875, 1963.

(*4*) Gross, L.: Serial cell-free passage of a radiation-activated mouse leukemia agent. Proc Soc Exp Biol Med 100: 102–105, 1959.

(*5*) Lieberman, M., and Kaplan, H. S.: Leukemogenic activity of filtrates from radiation induced lymphoid tumors of mice. Science 130: 387–388, 1959.

(*6*) Ageenko, A. I.: Leukemogenic agent isolated from irradiated C57BL mice. Probl Hematol 2: 8–13, 1962.

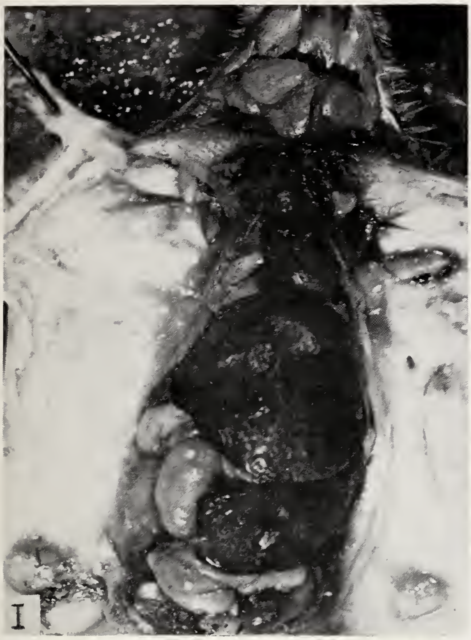
Plate 84

Figure 1.—Leukemia in C57BL/10ScSn mouse killed 17 months after inoculation. The second cell-free passage.

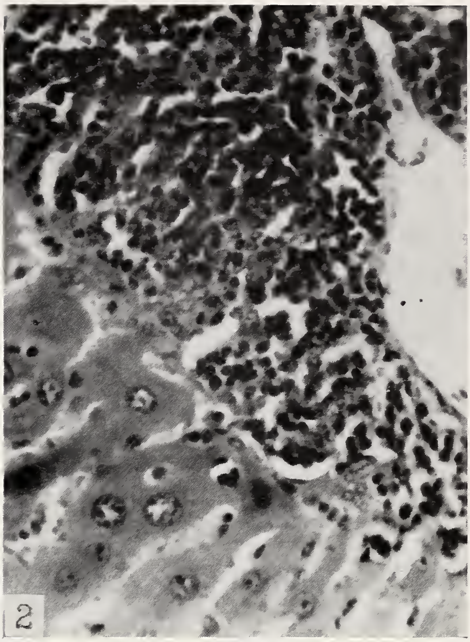
Figure 2.—A microscopic section of the liver of CC57W mouse killed 15.5 months after inoculation. First cell-free passage. Perivascular leukemic infiltration. Hematoxylin and eosin. × 420

Figure 3.—A microscopic section of the kidney of CC57W mouse killed 9 months after infection. Second cell-free passage. Diffusive leukemic infiltration. Hematoxylin and eosin. × 420

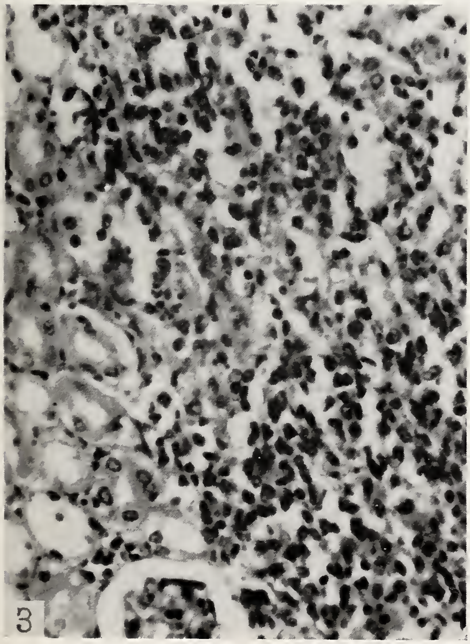
Figure 4.—A lymph node of CC57W mouse killed 4.5 months after inoculation. Third cell-free passage. Azure-eosin. × 1,350



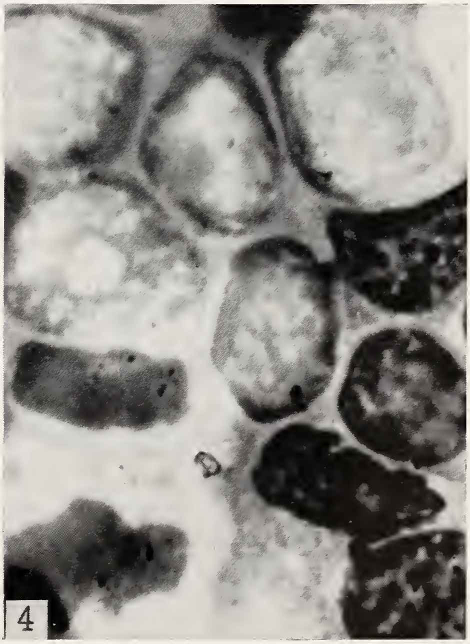
1



2



3



4

MURINE LEUKEMIA VIRUSES—THEIR DIFFERENTIATION AND CLASSIFICATION

Chairman: John B. Moloney

Are the Common Forms of Spontaneous and Induced Leukemia and Lymphomas in Mice Caused by a Single Virus?[1, 2]

Ludwik Gross, *M.D.*, *Cancer Research Unit, Veterans Administration Hospital, Bronx, New York*

To clarify the problem of viral etiology of mouse leukemia, it is necessary to consider certain fundamental observations and also to define more precisely the terminology used. Leukemia has been observed to develop spontaneously in mice, particularly in certain inbred lines, but it can be induced also by X irradiation, carcinogenic chemicals, or hormones (*1*). The conventional and widely used term "mouse leukemia" is not precise, however, and can be employed only in its broad meaning; theoretically, this term implies a condition of the blood characterized by increased number of white cells in peripheral blood circulation. In reality, however, when this term refers to the disease occurring in mice, a more precise designation, for most cases, would be "disseminated lymphosarcomatosis." In most instances the blood is not significantly affected, except for occasional presence of abnormal white cells, usually of the lymphatic series, and a gradually progressing anemia. The usual prominent feature of this disease in mice is a large thymic lymphosarcoma, and there is often a concomitant enlargement of the spleen—due to lymphoid tumor infiltration—and frequently of the liver. In fully developed disease, multiple lymphoid tumors replace the cervical, axillary, inguinal, and also mesenteric lymph nodes. Sometimes, however, the disease also involves the blood. Examination of the peripheral blood of such leukemic mice reveals an increased number of white blood cells, and also the presence of abnormal cells of either the lymphatic or myeloid series, and their precursors, as well as a gradually progressing anemia. The forms of mouse leukemia vary and include lymphatic, stem cell, and myeloid leukemia, also, less frequently, monocytic leukemia and chloroleukemia, and rarely the erythroblastic form. Most animals with true leukemia, involving blood pathology, show infiltration of internal organs with leukemic cells and have multiple lymphosarcomas, involving usually not only the

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Aided, in part, by grants from the Damon Runyon Memorial Fund, and the American Cancer Society.

thymus but also the spleen, lymph nodes, liver, and a variety of other organs. Reticulum cell sarcomas are less common, but also occur, particularly in mice of certain strains. Hodgkin's disease-like lesions are of considerable interest and can also be found at least in some organs and in some animals.

An animal which may at first develop a localized lymphosarcoma, usually in the thymus, may gradually develop additional lymphosarcomas in spleen, lymph nodes, liver, etc. Eventually the peripheral blood may show an increased number of white cells, as well as presence of abnormal blood cells. Thus, in the same host, one form may gradually change into another. Moreover, either lymphatic or myeloid leukemia may change on transplantation from one host to another into stem cell leukemia or eventually into a generalized lymphosarcomatosis. It is now quite apparent that this wide variety of leukemia and lymphomas observed in mice represents pathologic manifestations of different forms of essentially the same disease (*2–5*). Most of these various forms of mouse leukemia, particularly the disseminated lymphosarcomatosis, develop spontaneously in certain leukemic inbred lines of mice, such as AK or C58, and can also be induced in mice of low leukemic strains by total body X irradiation, certain carcinogenic chemicals, or hormones.

ISOLATION OF MOUSE LEUKEMIA VIRUS AND DEVELOPMENT OF A "FIXED VIRUS" STRAIN

In 1951 it was demonstrated that leukemia developing spontaneously in mice of certain strains of mice, such as AK, is caused by a filterable virus transmitted from one generation to another (*6*). However, the initial method of transmitting mouse leukemia by filtrates prepared from organs of AK donors with spontaneous leukemia was frequently disappointing and time-consuming. This was because of the relatively low potency of such extracts. This situation changed fundamentally when a highly active leukemogenic virus was isolated from some of the organ extracts tested and then passed serially through newborn mice. This virus strain, designated "Passage A" (*7*), is comparable to Pasteur's "fixed virus" of rabies, as contrasted with the "street" or "wild" virus encountered in animals developing disease spontaneously.

HOST RANGE

In the initial experiments, when filtrates prepared from spontaneous mouse leukemia of relatively low potency were employed, the virus was found to have a relatively narrow range of host-specificity. However, when in more recent experiments substantially more potent virus filtrates were employed for bioassay, the virus showed a much broader host range

(*5*). Mice of several inbred lines were found to be susceptible; furthermore, suckling rats of several strains were also highly susceptible to the leukemogenic action of Passage A virus (*8*).

FORMS OF LEUKEMIA AND LYMPHOMAS INDUCED WITH THE VIRUS

At first it was thought that the Passage A virus could induce in mice only the lymphatic form of this disease, manifested most frequently by the development of disseminated lymphosarcomas. Recently, however, it became clear that the same virus is capable of inducing a variety of different forms of leukemia and lymphomas in mice and rats (*2–5*, *8*). It is true that the most frequent form is that of a generalized lymphosarcoma, involving predominantly the thymus, and usually also the spleen, liver, and peripheral lymph nodes, with no significant changes in peripheral blood morphology. However, acute lymphatic leukemia, involving the blood morphology, as well as stem cell and myelogenous leukemias, occasionally, under certain experimental conditions also the more unusual forms, such as monocytic-like leukemia, chloroleukemia, and, on rare occasions even the erythroblastic form, could be induced in mice with the same virus (*3*). Lymphosarcomas were found in practically all animals; reticulum cell sarcomas and Hodgkin's disease-like lesions developed less frequently, but were also observed in animals of certain strains. Genetic susceptibility influenced to a considerable extent the form of leukemia developing in mice or rats following inoculation of the virus (table 1).

TABLE 1.—Forms of spontaneous or passage A virus-induced leukemia developing in mice and rats

Strain of mice or rats	Leukemia induced with	Number of mice or rats with leukemia	Lymphatic leukemia total incidence* (%)	Lymphatic "aleukemic" leukemia incidence† (%)	Stem cell leukemia incidence (%)	Myeloid leukemia incidence (%)
Mice						
AK	Spontaneous	100	92	63	5	3
AK × C57BR F_1 hybrids	Spontaneous	88	68	39	9	23
C58	Spontaneous	44	82	52	2	16
C3Hf	Passage A	100	87	64	12	1
C57BR	Passage A	29	63	34	34	3
BALB/c	Passage A	53	36	32	26	38
Rats						
S-D	Passage A (mouse)	44	45	34	48	7
S-D	Passage A (rat-adapted)	38	58	39	37	5

*Total incidence of "lymphatic leukemia" includes also the "aleukemic" group.

†With no significant leukemic changes in peripheral blood morphology. Most mice in this group had multiple lymphosarcomas, a few had thymic lymphomas only.

To some degree, the form of induced leukemia could also be influenced by thymectomy. In mice (*3*) and also in rats (*8*) the incidence of myeloid leukemia was higher in thymectomized animals.

The variety of forms of leukemias and lymphomas induced in mice with the "Passage A" virus reproduces quite accurately the spectrum of similar forms of this disease that develop in mice spontaneously, or are induced by radiation, hormones, or carcinogenic chemicals. The fact that all the conventional forms of leukemia and lymphomas observed in mice can be induced with a single virus is of considerable importance, and should be considered when an attempt is made to clarify the problem of the etiology of mouse leukemia, and of a classification of the different strains of mouse leukemia viruses isolated during the preceding decade.

ISOLATION OF MOUSE LEUKEMIA VIRUS STRAINS FROM DIFFERENT SOURCES

As soon as the experimental conditions were determined under which a leukemogenic virus could be isolated from organs of leukemic mice, and the importance of using newborn mice for bioassay was emphasized, it gradually became a routine procedure to follow the established technique and to isolate leukemogenic viruses from leukemic mouse tissues. Later, it also became apparent that a leukemogenic virus could be isolated not only from organs of mice with spontaneous leukemia but also from mice that developed radiation-induced leukemia (*9*). Normal mouse embryos from mice of high-leukemic AK and C58 inbred strains also could be used as source material for the isolation of the virus (*1*, *6*). Moreover, potent mouse leukemia virus strains could be isolated from nonleukemic transplanted mouse tumors, such as certain sarcomas and carcinomas (*1*, *10*, *11*).

HOW MANY DIFFERENT VIRUSES CAUSING MOUSE LEUKEMIA?

As soon as it became apparent that either lymphatic, myelogenous, or stem cell leukemia, as well as lymphosarcomas, reticulum cell sarcomas, and Hodgkin's disease-like lesions, could be induced in mice and rats with a single virus, the possibility had to be considered that the presumably different forms of leukemia induced in mice and rats with virus strains, isolated from different sources, may be caused by the same virus, or at best by one of its close variants (*12*).

It should be stressed that most mouse leukemia virus strains, even though isolated from different sources, such as from spontaneous mouse leukemia (*6*), radiation-induced leukemia (*9*), or different transplanted mouse tumors (*10*, *11*), are essentially indistinguishable in their morphology, and in their biological, physical, and pathogenic properties (*12*). When inoculated into suckling mice of susceptible inbred lines, these virus strains induce one of the conventional forms of mouse leukemia, in most instances lymphatic leukemia manifested by the development of generalized lymphosarcomas, usually affecting also the thymus, with or without concomitant involvement of blood morphology, and less frequently stem cell or myelogenous leukemia. Such virus strains may induce morphologically distinct forms of leukemia and lymphomas if tested in animals of different genetic susceptibility; when, however, they are studied under identical experimental conditions they induce essentially the same disease. Briefly, the different virus strains isolated from various sources and thought to represent distinct viruses actually induce the same disease, the form depending to a considerable extent on the genetic susceptibility of the host employed for bioassay.

MOUSE LEUKEMIA VIRUS TYPE A

It may be premature to state definitely that all conventional forms of mouse leukemia and lymphomas are caused by a single virus. However, on the basis of experimental data now available it is reasonable to assume that most of the individual isolations of leukemogenic viruses in mice represent recoveries from different sources of the same virus or at best one of its closely related strain variants, any of which may induce in mice the same disease. Even though some of these virus strains may show minor pathogenic or immunological differences, it is reasonable to assume they all represent basically the same virus. Apparently, it would be useful to designate this basic mouse leukemia virus by a symbol; for that reason it is suggested that the mouse leukemia virus and its related variants, which induce in mice of the conventional forms of mouse leukemia, be designated as the "mouse leukemia virus type A."

SOME OF THE PRINCIPAL PHYSICAL AND PATHOGENIC PROPERTIES OF THE MOUSE LEUKEMIA VIRUS TYPE A

The following properties were determined on the basis of studies carried out with the "Passage A" virus (*2*, *4*, *5*, *7*, *8*, *12*). The virus is a spherical particle about 100 mμ in diameter and can be found, on electron microscopic examination of organs of leukemic mice and rats, in the cytoplasm or in the intercellular spaces of virus-infected cells. The virus is thermolabile and can be inactivated after heating to only 50° C for 30 minutes. Furthermore, the virus is also sensitive to *in vitro* exposure to ethyl-ether in experiments performed under standard bacteriological conditions, *i.e.*, mixing the virus filtrate in proportions containing at least a 20% volume of ether.

TABLE 2.—Incidence of thymic lymphomas in leukemic mice and rats

Strain of mice or rats	Leukemia induced with	Number of mice or rats with leukemia	Number of animals with thymic lymphomas	Thymic tumor incidence (%)	Number of animals with thymic tumors only	Number of animals with lymphomas in spleen, liver, and lymph nodes
Mice						
AK	Spontaneous	97	97	100	4	93
C58	Spontaneous	44	38	86	—	44
C3Hf	Passage A	100	96	96	4	96
C57BR	Passage A	29	29	100	—	29
BALB/c	Passage A	53	53	100	2	51
Rats						
S-D	Passage A (mouse)	44	44	100	8	36
S-D	Passage A (rat-adapted)	38	37	97	5	33

The virus is pathogenic for suckling mice and rats of a variety of strains inducing usually generalized lymphosarcomas, with or without a concomitant involvement of peripheral blood morphology. Development of thymic tumors is a common and striking feature in most of the mice and rats developing leukemia as a result of the pathogenic action of this virus (table 2).

SERUM NEUTRALIZATION TESTS

This discussion would be incomplete without a brief reference to serum neutralization tests which are of considerable importance when an attempt is made to identify a virus. It should be stressed that the mouse leukemia virus has only a limited antigenic potential. Nevertheless, a specific, although only relatively potent immune serum can be obtained from rabbits following several consecutive injections of the Passage A mouse leukemia virus filtrate, preferably mixed with Freund adjuvant. Serum obtained from such rabbits, even though it has only a limited neutralizing potential, can be useful, since it will neutralize the mouse leukemia virus *in vitro*, provided that an undiluted serum, or a 1:2 dilution of the serum, is employed, and provided, furthermore, that the dilution of the virus filtrate employed for the neutralization test is of proper titer. Neutralization occurs only under carefully performed quantitative conditions. It should be emphasized that when a filtrate containing an unknown and possibly high concentration of a virus is mixed with a heterologous immune serum, neutralization may not occur even when the immune serum contains specific antibodies. This difficulty may be due to the low potency of the serum and the high titer of the virus present in routinely prepared filtrates. For that reason, preliminary titration of the virus filtrate is imperative before the serum neutralization tests are performed. This was

TABLE 3.—Neutralization of Passage A mouse leukemia virus* and Moloney strain by immune Passage A rabbit serum†

		Passage A virus			Moloney strain		
Serum from rabbits immunized with	Serum dilution	Virus filtrate dilution	Number of mice inoculated§	Number developing leukemia‡	Virus filtrate dilution	Number of mice inoculated§	Number developing leukemia‡
Exp. 1							
Passage A virus (with adjuvant)	Undiluted	10^{-3}	8	0	10^{-3}	11	1
Normal mouse organs (with adjuvant)	Undiluted	10^{-3}	9	9	10^{-3}	13	13
Saline controls		10^{-3}	6	6	10^{-3}	3	2
Exp. 2							
Passage A virus (with adjuvant)	Undiluted	10^{-3}	8	0	10^{-3}	5	0
Passage A virus (with adjuvant)	1 : 2	10^{-3}	5	0	10^{-3}	5	1
Normal mouse organs (with adjuvant)	Undiluted	10^{-3}	4	4	10^{-3}	7	7
Normal mouse organs (with adjuvant)	1 : 2	10^{-3}	7	6	10^{-3}	11	11
Saline controls		10^{-3}	10	10	10^{-3}	8	8

*Virus filtrate mixed 1:1 with serum, incubated at room temperature 30 minutes, and then at + 4° C for 4 hours.

†Serum inactivated at 56° C 30 minutes.

‡Leukemia developed at ages varying from 3 to 5 months. Surviving mice which did not develop leukemia were observed for periods of time varying from 9 to 13 months.

§Less than 6-day-old suckling C3Hf mice inoculated intraperitoneally with 0.3 ml.

demonstrated in recent experiments (*13*) in which it was observed that neutralization of the mouse leukemia virus occurred only when 10^{-3} dilution of the virus filtrate was mixed with an equal amount of undiluted immune serum. When a higher concentration of virus filtrate or a less potent serum was used, neutralization was less pronounced or did not occur at all.

Recently, we have studied one of the more potent leukemogenic virus strains isolated by Dr. John B. Moloney from a transplanted mouse sarcoma (*11*). As described in more detail elsewhere (*12*), this virus strain is indistinguishable from the Passage A virus in its physical and pathogenic properties, except for a slightly higher titer. In a recent study it was demonstrated (*13*) that under proper quantitative conditions, both Passage A virus and the Moloney strain could be neutralized *in vitro* by the same specific serum obtained from rabbits immunized by repeated injections of Passage A virus filtrate (table 3).

A considerable number of individual isolations of leukemogenic viruses from different sources in mice has been reported from many laboratories. It is possible that serum neutralization tests carried out under quantitative conditions with at least some of these virus strains would also show an immunological relationship similar to that described to exist between the Passage A virus and the Moloney strain. On the other hand, it should not be surprising to find some possibly minor immunological differences between some of the mouse leukemia virus strains thus far isolated. Immunological and other differences among individual virus strains in many other virus diseases are quite common. Variants of the Rous sarcoma virus or of the polyoma virus have been described. Such variants may be distinct antigenically; some of them may even show differences in their pathogenic potency, evident on inoculation tests or in tissue culture.

FRIEND VIRUS

The current difficulties in clarification and classification of the mouse leukemia viruses have been increased by the introduction into the study of experimental mouse leukemia of a progessive disease of the hematopoietic system developing in mice after inoculation of the Friend virus. This curious disease seems to be a true "laboratory disease" and appears to be exceedingly rare under natural life conditions, if it occurs spontaneously at all. The disease can be, however, readily induced in the laboratory by inoculating susceptible mice with a virus which was isolated originally from a Swiss mouse by Dr. Charlotte Friend (*14*).

Following inoculation of filtrates prepared from spleens and livers of donor mice with Friend-virus-induced disease into 3- to 4-week-old mice of several susceptible strains, such as DBA/2, BALB/c, C3Hf, or Swiss, progressive enlargement of spleen and liver is induced after a relatively short latency of only a few weeks. In susceptible mice, the enlargement of spleen and liver is the main macroscopic picture of the induced disease; the spleen may become enormously enlarged and filled with blood, like a balloon. There are no thymic tumors, and the peripheral lymph nodes are not enlarged. This is in striking contrast to most of the conventional forms of mouse leukemias. It should be emphasized that the Friend disease has no apparent relation to the presence or absence of thymus. The course of the disease induced with the virus in thymectomized mice was the same as that in nonthymectomized controls.

The peripheral blood smear reveals a characteristic picture: There is evidence of considerable anemia and nucleated red cells are common; most striking, however, are innumerable "smudge cells" appearing among various cells of the myeloid series. On microscopic examination of liver and spleen there is considerable infiltration with abnormal white cells with areas of destruction and necrosis; some of the other organs may also show infiltration with abnormal cells, but to a considerably lesser extent.

The disease may become chronic, with enlargement of spleen persisting over long periods. In most instances, however, the disease is progressing rapidly and the animals die within 2 or 3 months after inoculation. The latency and survival time depend on the genetic susceptibility of the inoculated mice, also on age at inoculation, and dose of the injected virus. Inoculation of the virus filtrate into newborn or suckling mice of susceptible strains induces a rapidly progressing disease of short duration.

The disease induced by the Friend virus probably belongs to the broad group of leukemias. This blood disease is, nevertheless, very different from any of the usual forms of leukemia, occurring spontaneously in mice and other animals, or induced by radiation, chemicals, or hormones. Some of the basic differences between conventional forms of leukemia and the Friend-virus-induced disease in mice are as follows:

	Mouse leukemia	Friend virus-induced disease
Spontaneous occurrence	Common in certain strains	Not observed
Induction by X rays, chemicals, or hormones	Common	None
Effect of thymectomy	Considerable delay or prevention	None
Development of thymic tumors	Over 90%	None
Development of disseminated lymphosarcomas	Common	None
Enlargement of peripheral lymph nodes due to development of lymphoid tumors	Common	Not observed
Peripheral blood involvement	Less than 50%	100%
Anemia	Usually moderate	Very pronounced

RAUSCHER VIRUS STRAIN

The leukemic virus strain isolated recently by Dr. Frank J. Rauscher (*15*) induces a disease apparently identical with that resulting from inoculation of the Friend virus. Following inoculation into either suckling or 3- to 4-week-old mice of strains such as C3H or BALB/c, the Rauscher virus strain induces, after a short incubation of only a few weeks, a rapidly progressing enlargement of spleen and liver and the development of a considerable and progressing anemia. As in the case of Friend disease, the enormous and blood-filled spleen may present the most impressive pathologic picture. The white blood cell count is considerably increased and the peripheral blood smears show pronounced anemia, many nucleated red cells, and characteristically the presence of very large numbers of "smudge cells" among a variety of cells of the myeloid series in different phases of maturation. Thus, the peripheral blood picture in fully developed disease is indistinguishable from that induced in susceptible mice with the Friend virus. Microscopic examination of the liver and spleen of such animals reveals a pathologic picture very similar to that observed in mice with Friend-virus-induced disease.

Under certain experimental conditions, however, inoculation of the Rauscher virus strain may result in the development of thymic or generalized lymphosarcomas, or of another conventional form of mouse leukemia, such as lymphatic, stem cell, or myeloid leukemia. This may occur when newborn mice of certain strains—such as C57BL or C57BR—or suckling rats are inoculated with the Rauscher virus strain. In this respect the Rauscher virus strain is also similar to the Friend virus, since under certain experimental conditions the Friend virus may also induce lymphatic leukemia, including a striking development of thymic lymphosarcomas and replacement of peripheral lymph nodes by characteristic lymphoid tumors (*16*), so typically seen in spontaneous or Passage A virus-induced leukemia. This may occur when the Friend virus filtrate is inoculated into mice which are relatively resistant to the induction of the Friend disease, such as mice of strain C57BR (*16*) or into rats (*16*, *17*). Although lymphatic leukemia could also be induced with the Friend virus in mice of certain strains, we have had the impression in our studies that the Rauscher virus strain more readily and more frequently induces lymphatic and other forms of conventional mouse leukemia than does the Friend virus (*16*).

It would be possible to explain this multiplicity of forms of disease induced with the Rauscher virus strain, and to a lesser extent also with the Friend virus, by an assumption that both represent a single multipotent virus capable of inducing either: (*a*) a hepatomegaly syndrome with characteristic pathologic features, including the striking blood picture and microscopic manifestations of the Friend disease; or (*b*) a conventional form of mouse leukemia—such as lymphatic, stem cell, or myeloid leukemia—most frequently associated with the development of a thymic lymphosarcoma, and a generalized lymphosarcomatosis, involving liver, spleen, peripheral lymph nodes, etc., *i.e.*, one of the usual forms of mouse leukemia induced with the mouse leukemia virus type A. On the other hand, it would be also possible to assume that what is now considered to be the Rauscher or Friend virus may actually represent a mixture of two distinct viruses. It is conceivable that a filtrate prepared from spleens and livers of mouse donors with either the Rauscher- or the Friend-virus-induced disease may contain two distinct leukemic viruses, the Friend virus and also the mouse leukemia virus type A.

TWO DIFFERENT ONCOGENIC VIRUSES IN ONE EXTRACT?

When a virus is harvested from diseased organs of an animal host, the possibility of an accidental contamination with another virus carried by the same host must be considered. Even after several differential centrifugations of the organ extracts prepared, the final supernatant fluid may not necessarily represent anything comparable to a pure and uncontaminated culture of a single virus. More often it will contain also one or more additional viruses, possibly in lower concentration, or perhaps in trace quantities only, even though only one of them might have induced pathologic manifestations in the donor host. The presence of such contaminating "passenger" viruses might not have been suspected at the time the organs were removed for the preparation of the extract, but may be revealed later on, accidentally, on bioassay.

ACCIDENTAL DISCOVERY OF THE "PAROTID TUMOR" (POLYOMA) VIRUS AS A CONTAMINANT OF LEUKEMIC MOUSE EXTRACTS

It may be of interest to recall at this point the early studies on mouse leukemia which were complicated by the presence in leukemic mouse extracts of another unrelated virus, inducing salivary gland tumors. In the initial studies, when the mouse leukemia virus was first isolated from organs of AK mice with spontaneous leukemia, some of the extracts prepared from such organs, inoculated into newborn C3H mice, induced parotid gland tumors instead of leukemia (*18*). A few animals developed both leukemia and parotid tumors. The possibility had to be considered that a single virus could have been responsible for the induction of either leukemia or parotid gland tumors. It was soon realized, however, that extracts prepared from leukemic mouse tissues contained two different viruses, one causing leukemia, and another causing parotid gland tumors (*18*). The parotid tumor virus, later propagated by Stewart, Eddy, and Borgese in tissue culture (*19*), eventually revealed its full pathogenic potential and was designated the "polyoma" virus (*20*). The mouse leukemia and the parotid tumor viruses, even though initially present simultaneously in the same filtrates, could readily be separated by ultracentrifugation or by differential heating, since the mouse leukemia virus was found to be larger and more susceptible to heat than the parotid tumor virus (*18*).

In general, however, the separation of two different viruses present in the same filtrate may be fraught with considerable difficulties, particularly if such viruses have similar physical and biological properties.

A MIXTURE OF FRIEND VIRUS AND OF MOUSE LEUKEMIA VIRUS TYPE A?

Let us consider the possibility that the Rauscher virus strain (and actually, though to a lesser extent, also the Friend virus filtrate) consists of a mixture of the Friend virus, in a relatively high titer, and of the mouse leukemia virus type A (*i.e.*, Passage A virus), the latter in a relatively lower concentration. Following inoculation of such a filtrate into animals susceptible to both viruses, either form of the disease could be induced. However, the Friend virus induces disease after a relatively shorter latency and for that reason, when susceptible mice are inoculated, they usually develop, and die from, the Friend form of disease before they have an opportunity to develop lymphatic leukemia, or another form of leukemia caused by the mouse leukemia virus type A, the latter requiring a considerably longer latency period.

On the other hand, if the mixture of the two viruses is inoculated into mice of certain inbred strains—such as C57BL or C57BR—or into suckling rats, which are relatively resistant to the induction of the Friend disease, one of the conventional forms of mouse leukemia—such as lymphatic, stem cell, or myeloid leukemia—may develop as a result of the pathogenic action of the mouse leukemia virus type A present in the filtrate. Characteristically, most of these animals develop thymic lymphosarcomas and, frequently, also typical enlargement of peripheral lymph nodes, generally absent in mice with Friend-virus-induced disease.

In some instances, a "mixed form" representing both the Friend disease and one of the conventional forms of mouse leukemia, such as thymic lymphosarcomas, can also be induced in the same host. Pathologic manifestations which could be attributed to both viruses have been observed quite frequently in our laboratory in some of the mice, and on rare occasions also in rats inoculated with the Rauscher virus strain.

ATTEMPT TO ELIMINATE FROM THE RAUSCHER VIRUS STRAIN EITHER THE FRIEND VIRUS OR THE LYMPHATIC LEUKEMIA VIRUS COMPONENT

Results of recent experiments in our laboratory are consistent with the assumption that the Rauscher virus strain actually consists of a mixture of two viruses. In preliminary experiments, an attempt was made to neutralize *in vitro* the Friend virus, presumably present in the Rauscher virus strain, by a specific immune serum. The Rauscher virus was mixed with immune serum obtained from rabbits that had received several injections of Friend virus filtrates. The mixture was then inoculated into suckling and also into 3-week-old C3Hf mice. After Friend serum neutralization, the Rauscher virus strain filtrate was still able to induce lymphatic leukemia; however, its potency to induce Friend disease was considerably diminished (*16*).

These experiments are now repeated, employing a more potent immune rabbit serum prepared with the Friend virus, and with an effort to follow a more quantitative approach to the neutralization test. It is hoped that under more precise experimental conditions it will be possible to inhibit completely the Friend virus component of the Rausher virus strain.

In another series of experiments, still in progress, the Rauscher virus strain was passed serially through rats, *i.e.*, through a species relatively resistant to the Friend disease. Following inoculation of the Rauscher virus strain filtrate into suckling rats, leukemia, usually of the lymphatic form, was induced in practically all animals.

The virus was then harvested from the leukemic rat donors and inoculated, in the form of filtrates, into newborn rats. The Rauscher virus strain has been passed in this manner through 8 consecutive rat-to-rat passages, inducing in practically all of the inoculated animals typical leukemia, similar to that induced with the Passage A virus. Most of these rats developed thymic tumors and generalized lymphosarcomas, frequently lymphatic or stem cell leukemia, and in some instances the myelogenous form. From each consecutive rat-to-rat passage, an attempt was made to inoculate the rat-adapted virus back into suckling, or 3- to 4-week-old C3Hf mice known to be uniformly susceptible to the Friend disease. After 4 to 6 consecutive rat-to-rat passages, the Rauscher virus strain lost much of its ability to induce a typical Friend disease. After inoculation into either suckling, or into 3- to 4-week-old, C3Hf mice, such rat-adapted virus induced either typical lymphatic leukemia or a "mixed" form, manifested by the development of thymic lymphosarcomas and also some of the characteristic features, such as the blood picture, of the Friend disease; only exceptionally, however, did such a virus induce a typical Friend disease. After 8 consecutive rat-to-rat passages, the Rauscher virus strain apparently lost its characteristic potency to induce symptoms of Friend disease, but retained fully its ability to induce lymphatic leukemia. When filtrates prepared from organs of such leukemic rat donors were inoculated into suckling C3Hf mice, thus far only thymic lymphosarcomas and other forms of lymphatic leukemia developed in some of the inoculated mice, with no evidence of Friend disease. These experiments are still in progress; additional passages of the Rauscher virus strain through rats are being made to determine whether a better separation of the two virus fractions could be obtained.

NEUTRALIZATION *IN VITRO* OF THE LYMPHATIC LEUKEMIA VIRUS COMPONENT OF THE RAUSCHER VIRUS STRAIN WITH IMMUNE PASSAGE A VIRUS SERUM

Since the "rat-adapted" Rauscher virus strain retained fully its potency to induce thymic and generalized lymphosarcomas, we attempted to determine whether this pathogenic potency of the Rauscher virus strain could be inhibited by an immune serum obtained from rabbits immunized with the Passage A virus. In a preliminary experiment, a Rauscher virus strain that had been passed through 8 consecutive rat-to-rat passages was employed. The virus filtrate was prepared from thymic tumors and spleens of leukemic rat donors, employing our method used routinely for the preparation of Passage A filtrates (*7*). A 10^{-3} dilution of the rat-adapted Rauscher virus strain filtrate was then mixed *in vitro* with an equal amount of undiluted immune serum obtained from rabbits that had received several consecutive injections of Passage A virus, harvested from leukemic C3Hf mice, with Freund adjuvant (*13*). In a simultaneous control experiment, a 10^{-3} dilution of the virus filtrate was mixed with an equal amount of undiluted serum obtained from rabbits that had received several injections of normal C3Hf mouse organs with Freund adjuvant (*13*). All sera were inactivated at 56° C for 30 minutes before neutralization tests. In a third group, as an additional control, the 10^{-3} virus filtrate dilution was mixed with an equal amount of physiological saline solution. The virus-serum mixtures, as well as the virus-saline control, were incubated at room temperature for 30 minutes, followed by incubation at +4° C for 3½ hours, and were then inoculated into suckling Sprague-Dawley rats. The results, summarized in table 4, indicate clearly that the Passage A immune rabbit serum inhibited completely the ability of the Rauscher virus strain to induce lymphatic leukemia after inoculation into suckling rats. There was no inhibiting effect of the normal mouse organ serum. Virus filtrate mixed with serum of normal mouse organ serum, as well as the virus filtrate diluted only, as a control, with equal amount of physiological saline solution, induced in all inoculated rats large thymic lymphosarcomas and generalized lymphosarcomas in spleens,

TABLE 4.—Neutralization *in vitro* of lymphatic leukemia component of Rauscher virus strain* with immune Passage A virus serum

Antigen employed for preparation of rabbit serum	Serum dilution	Virus filtrate dilution	Number of rats inoculated†	Number of rats developing leukemia‡	Leukemia incidence (%)	Average age leukemia developed (months)
Passage A virus harvested from mice (with adjuvant)	Undiluted	10^{-3}	5	0	0	—§
Normal mouse organs (with adjuvant)	Undiluted	10^{-3}	6	6	100	<5
Saline controls		10^{-3}	6	6	100	<4

*Rauscher virus strain after 8 consecutive rat-to-rat passages, harvested from thymic tumors and spleens of leukemic rat donors. Virus filtrate mixed 1:1 with undiluted serum, incubated at room temperature for 30 minutes, then for 3½ hours at +4° C.

†Less than 7-day-old suckling Sprague-Dawley rats were inoculated intraperitoneally with 0.6 to 0.8 ml.

‡Lymphatic leukemia with development of large thymic lymphoid tumors and generalized lymphosarcomas involving spleen, liver, and lymph nodes.

§Surviving animals observed 9 to 10 months for development of leukemia.

livers, and other organs, with or without concomitant involvement of peripheral blood morphology.[3]

Results of these preliminary experiments are consistent with the assumption that the Rauscher virus strain actually represents a mixture of Friend virus and of the mouse leukemia type A (*i.e.*, Passage A) virus, and that, under certain experimental conditions, these two different virus fractions can be separated or inhibited by a specific immune serum directed either against the Friend virus or the mouse leukemia virus type A component.

ETIOLOGY OF MOUSE LEUKEMIA

It is apparent from this review that the differentiation and classification of mouse leukemia viruses now encounter many difficulties. These difficulties are due mostly to the fact that: (*a*) The term "mouse leukemia" is not clearly and adequately defined and covers a considerable variety of different forms of this disease; (*b*) most of the conventional forms of mouse leukemia can be induced with a single virus; (*c*) leukemogenic viruses can be isolated readily from a variety of sources, not necessarily from organs of leukemic mouse donors, but also from normal mouse tissues, and from nonleukemic mouse tumors such as certain transplanted sarcomas and carcinomas; (*d*) most of the mouse leukemia virus strains thus far isolated from different sources have identical physical and biological properties and identical morphology; they have a similar host range, and similar pathogenic properties on bioassay, and induce one of the conventional forms of mouse leukemia; (*e*) finally, the difficulty in differentiation of mouse leukemia viruses has been considerably increased by the introduction into experimental pathology of the Friend virus, which in susceptible mice causes a disease of the hematopoietic system, principally involving spleen, liver, and blood morphology, but differing fundamentally from the conventional forms of mouse leukemia.

It is quite possible, however, that clarification in the field of mouse leukemia viruses will be eventually forthcoming. Thymus seems to be intimately related to the conventional forms of mouse leukemia—such as disseminated lymphosarcomas and reticulum cell sarcomas, as well as lymphatic, stem cell, and to some degree also myelogenous leukemia—whereas it is not involved in the pathology of certain other diseases of the hematopoietic system, such as the Friend disease. The latter disease may belong to the group of leukemias—at least in principle, and in the broad meaning of this term—but represents a fundamentally different syndrome. As for the large numbers of different virus strains isolated from many sources, and causing on bioassay one of the conventional forms of mouse leukemia, serological identification by the classical *in vitro* neutralization method may offer considerable promise in future studies, provided, however, that precise quantitative methods are employed for such neutralization tests.

---

[3] As this manuscript goes to press, results of a second experiment, carried out under similar conditions as those reported in table 4, became available, as follows:

Seven suckling Sprague-Dawley rats were inoculated with a 10^{-3} dilution of rat-adapted (after 9 consecutive rat-to-rat passages) Rauscher virus strain filtrate, mixed *in vitro* with undiluted immune serum obtained from rabbits that had received injections of passage A virus. Only 1 of the 7 injected rats developed leukemia at 8 months of age; the surviving animals in this experiment were observed for 9 to 10 months, and remained in good health. In a simultaneous control experiment, 6 suckling rats were inoculated with a 10^{-3} dilution of the virus filtrate mixed *in vitro* with an equal amount of undiluted serum obtained from rabbits that had received injections of normal mouse organs. Five of the 6 inoculated rats developed leukemia after a latency varying from 3–5 months. In another control group, 4 suckling rats were inoculated with a 10^{-3} dilution of the same Rauscher virus strain filtrate mixed *in vitro* with physiological saline solution, and all developed leukemia after a latency varying from 2–5 months.

It is possible to speculate, on the basis of results of certain preliminary experiments thus far performed, that most of the individual isoluations of leukemogenic viruses in mice represent recoveries from different sources of the same leukemia virus, or at best one of its closely related variants.

SUMMARY

Mouse leukemia Passage A virus can induce in mice and rats all the conventional forms of leukemia, usually associated with a thymic tumor and generalized lymphosarcomas, such as lymphatic, stem cell, and, less frequently, myelogenous leukemia. The same virus can also induce reticulum cell sarcomas and Hodgkin's disease-like lesions. The difficulty in differentiation and identification of the mouse leukemia viruses has been increased by the introduction into experimental pathology of a distinctive blood disease, belonging to the broad group of leukemias, which can be induced in mice by inoculation of the Friend virus. Moreover, a filtrate prepared from leukemic mouse tissues may contain both the Friend virus and the conventional mouse leukemia virus. Such a filtrate, on bioassay, may induce in susceptible mice either the Friend disease or the usual form of mouse leukemia, such as thymic or generalized lymphosarcomas.

In recently published experiments, an immune rabbit serum prepared with Passage A virus neutralized *in vitro* the Moloney strain of the mouse leukemia virus. In a preliminary experiment reported in this study, the lymphatic leukemia component of the Rauscher virus strain was also neutralized *in vitro* by an immune Passage A serum obtained from rabbits. Results of these neutralization tests are consistent with the assumption that the mouse leukemia virus, originally isolated from spontaneous AK mouse leukemia, represents the prototype of the virus responsible for the induction of the common forms of lymphatic and other conventional forms of leukemia in mice (mouse leukemia virus "type A"). It is also possible that what is now considered to be the "Rauscher virus" actually represents a mixture of the Friend virus and of the mouse leukemia virus type A.

REFERENCES

(*1*) Gross, L.: Oncogenic Viruses. Oxford and New York, Pergamon Press, 1961, 393 pp.

(*2*) ———: Properties of a virus isolated from leukemic mice, inducing various forms of leukemia and lymphomas in mice and rats (Bertner Lecture). *In* Viruses, Nucleic Acids, and Cancer. Baltimore, Williams & Wilkins Co., 1963, pp 403–426.

(*3*) ———: Development of myeloid (chloro-) leukemia inthymectomized C3H mice following inoculation of lymphatic leukemia virus. Proc Soc Exp Biol Med 103 : 509–514, 1960.

(*4*) ———: Biological and pathogenic properties of a mouse leukemia virus. Acta Haemat (Basel) 23 : 259–275, 1960.

(*5*) ———: Pathogenic potency and host range of the mouse leukemia virus. Acta Haemat (Basel) 29 : 1–15, 1963.

(*6*) ———: Pathogenic properties, and "vertical" transmission of the mouse leukemia agent. Proc Soc Exp Biol Med 78 : 342–348, 1951.

(*7*) ———: Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proc Soc Exp Biol Med 94 : 767–771, 1957.

(*8*) ———: Serial cell-free passage in rats of the mouse leukemia virus. Effect of thymectomy. Proc Soc Exp Biol Med 112 : 939–945, 1963.

(*9*) ———: Serial cell-free passage of a radiation-activated mouse leukemia agent. Proc Soc Exp Biol Med 100 : 102–105, 1959.

(*10*) Graffi, A., Bielka, H., and Fey, F.: Leukämieerzeugung durch ein filtrierbares Agens aus malignen Tumoren. Acta Haemat (Basel) 15 : 145–174, 1956.

(*11*) Moloney, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J Nat Cancer, Inst 24 : 933–951, 1960.

(*12*) Gross, L.: How many different viruses causing leukemia in mice? Acta Haemat (Basel) 32 : 44–62, 1964.

(*13*) ———: Neutralization *in vitro* of mouse leukemia virus by specific immune serum. Importance of virus titration. Proc Soc Exp Biol Med 119 : 420–427, 1965.

(*14*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105 : 307–318, 1957.

(*15*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29 : 515–543, 1962.

(*16*) Gross, L.: Attempt at classification of mouse leukemia viruses. Acta Haemat (Basel) 32 : 81–88, 1964.

(*17*) Mirand, E. A., and Grace, J. T., Jr.: Induction of leukemia in rats with Friend virus. Virology 17 : 364–366, 1962.

(*18*) Gross, L.: A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. Proc Soc Exp Biol Med 83 : 414–421, 1953.

(*19*) Stewart, S. E., Eddy, B. E., and Borgese, N.: Neoplasms in mice inoculated with a tumor agent carried in tissue culture. J Nat Cancer Inst 20 : 1223–1243, 1958.

(*20*) Eddy, B. E., Stewart, S. E., and Berkeley, W.: Cytopathogenicity in tissue cultures by a tumor virus from mice. Proc Soc Exp Biol Med 98 : 848–851, 1958.

DISCUSSION

Dr. Siegler: I would like to comment on your statement that the Friend and Rauscher diseases are no "leukemias," and that they do not occur in man. Perhaps this is not so easily decided.

As to whether these diseases are "leukemias" is a matter of definition. They certainly have the unrestrained proliferation of primitive hematopoietic cells of the spleen, and a terminal phase of flooding of these cells into the peripheral circulation. This is all Virchow ever meant by the term "leukemia." Whether these proliferating cells are *autonomous* or not is irrelevant to the definition of leukemia, since to date no human leukemia has ever been shown to be autonomous.

Whether these diseases occur in man cannot be answered by the weight of present authority alone, since our information is gathered from traditionally oriented sources. This comes out of the very idea of "diagnosis," which is a dialectic choice between possible alternatives. When Metcalf, Furth, and Buffett (Cancer Res., 19: 52, 1959) sent slides of the Friend disease as unknowns to 16 pathologists, the diagnoses returned included reticulum cell sarcoma and leukemias of all types. Not one of these pathologists felt that it was not a hematopoietic neoplasm. Had the slides been prelabeled as a "virus-induced disease of mice" and then submitted for diagnosis, possibly such consensus would have been lacking. Actually, leukemias in humans have been described which strongly resemble these mouse diseases (di Guglielmo, Rev. Hematol. 1: 355, 1946). It is simply impossible to know the true incidence of such diseases in man, since they are at present not included among the permissible categories from which a diagnosis is made. We are simply too sophisticated to ever make the diagnosis of a mouse disease in a human.

Dr. Rappaport: I have studied human and murine leukemias and related diseases for many years, and I do not know of the existence of any human disease that would correspond to Friend's disease.

Dr. Tennant: I agree with Dr. Gross in general. I would limit my comment to lymphoid leukemias only because those are within my own experience.

Although I didn't specifically state so in my paper, what I was trying to say was that I felt all of these viruses, *i.e.*, those inducing lymphoid leukemias, were indeed the same and that the difference was perhaps the coating about the virus which changed its host range but not the nature of the disease induced. This is the sort of thing I am trying to prove currently. There is now quite a lot of evidence that such indeed may be the case.

I wanted to comment when Dr. Law presented his paper regarding vertical transmission and Dr. Gross took exception to the results. Maybe we need a unifying principle introduced here and there. I don't think there need really be disagreement, since the results were not obtained with the same virus material and not in the same system. It may be vertical transmission occurs when there is "establishment" of the virus in the host system, and the process of this establishment would, by definition, mean the acquisition of a host membrane coating (or of some modifying quality) from the "new" strain, so that thenceforth the virus would be readily passed from generation to generation in the manner both men described.

Dr. Friend: I don't want to get into the argument about the pathogenesis of the various virus-induced leukemias, but I wish to emphasize that as investigators we are interested in the "unfortunate accident" that happens to make cells neoplastic, so that any such model system is of extreme importance to us.

Dr. Upton: I would like to voice my admiration of Dr. Gross' work. As the chairman has said, Dr. Gross has opened up this whole field of murine leukemia virology, and as a result of his work, and complementary work in other laboratories, it is evident that, as he emphasized, the majority of the forms of leukemia we see in the mouse apparently can be produced by viruses. Unfortunately, the problem doesn't end here. We still are unable to control these diseases. We still do not know how viruses induce them, or whether in any given case the disease is, in fact, virus-induced, although it might be. I think it is to this latter series of questions that we must address ourselves now.

Murine Leukemia and Its Viruses [1, 2]

Marvin A. Rich,[3] *Department of Cell Biology, Albert Einstein Medical Center, York and Tabor Roads, Philadelphia, Pennsylvania*

As suggested earlier in this Conference, despite the accumulation of considerable data on neoplasia induced by a variety of stimuli, we have at the present time no significant insight into the specific mechanisms governing the conversion of controlled functional growth and development into that condition which we describe as neoplasia.

The demonstration by Ludwik Gross (*1*), of an unequivocal viral etiology for murine leukemia, focused attention on this experimental approach to an understanding of the mechanism of this one type of neoplasia. Since his finding there have been numerous reports describing viruses with leukemogenic potential for mice. There is little doubt that, based on criteria as set forth for the classification of other viruses such as morphology, susceptibility to physical and chemical agents, host specificity, and pathogenesis, all of the known leukemogenic agents of the mouse share many common characteristics. As a result, these agents have been described both as a family of closely related viruses (*2*) and as a single virus to be designated as "mouse leukemia virus type A" (*3*).

A brief perusal of the murine leukemia literature, or even the program of this Conference with its nomenclature of Friend, Rauscher, radiation leukemia, Moloney, and Rich viruses, evidences that the effective differentiation and taxonomic characterization of these agents have been limited to date by the unavailability of suitable studies, employing the immunological and *in vivo* assay techniques shown to be effective in the differentiation of other virus groups. Fortunately, this delay has not limited the usefulness of the murine viral leukemias as model systems for the study of various aspects of leukemogenesis.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by Public Health Service grant CA-06711 from the National Cancer Institute.

[3] National Institutes of Health Career Development Awardee (1-K3-CA-21773). I am indebted to Dr. Richard Siegler of this laboratory for pathological diagnosis, to Dr. John Moloney and Dr. Frank Rauscher of the National Cancer Institute for materials and advice, and to Dr. Lloyd Old, Sloan-Kettering Institute, New York, N.Y., for carrying out cytotoxicity tests. The excellent technical assistance of Rodney Clymer and Janice Geldner is acknowledged.

Some time ago we described the reproducible incidence of malignant lymphoma in ICR/Ha Swiss mice inoculated with nucleoprotein extracts of spleen from mice infected with Friend virus (*4*). Subsequently, we described the biological and some physiochemical characteristics of this virus and the disease or diseases initiated by it (*5*). The pathogenesis of this disease, described in detail by Siegler *et al.* (*6*), places it with the thymic lymphomas as represented by the Gross and Moloney leukemias—this in contrast to the *splenic* group, as in the diseases initiated by the Friend and Rauscher viruses. The virus, morphologically similar to the other murine leukemia viruses, induced lymphoid leukemia in a variety of mouse strains and in Osborne-Mendel rats (*5*). While leukemogenic activity was inversely proportional to age, ICR/Ha Swiss mice up to 6 months of age could be infected. Titers of 10^5 to 10^7 infectious units could be attained in BALB/c or ICR/Ha Swiss mice (*5*). To better understand the relationship existing between the various murine leukemia viruses we compared the Rich, Friend, Rauscher, and Moloney viruses using several immunological procedures.

MATERIALS AND METHODS

The experimental procedures employed in these experiments have been described previously (*5*).

RESULTS AND DISCUSSION

Vaccines

Formalinized vaccines prepared against Friend and Rich viruses—by a modification of the method suggested by Friend (*7*)—were administered intraperitoneally into small groups of ICR/Ha Swiss mice at 2, 3, and 4 weeks of age. At 5 weeks these animals and unvaccinated controls were challenged with a leukemogenic dose of Rich virus.

Table 1 shows that mice receiving either Rich or Friend virus vaccine had a reduced leukemogenic response to infection by the Rich virus.

TABLE 1.—Effect of Friend and Rich vaccines on subsequent infection by Rich virus

Group	Number leukemic* / Number inoculated	Mean latent period (days)†
No vaccine	4/5	115
Rich virus vaccine	1/5	138
Friend virus vaccine	1/5	175

*Swiss mice were challenged with 0.3 ml intraperitoneally of a 2×10^{-2} dilution of Rich virus.

†Time elapsed between inoculation and the appearance of palpable spleen and peripheral lymph nodes.

This was evidenced by reduced incidence and longer latent period. In other experiments not shown here, animals which were vaccinated beginning at 48 hours and challenged at 3 weeks of age were not significantly protected by either the Rich or Friend virus vaccines (*5*). The results of these studies suggested that those antigenic components involved in protection by formalinized virus vaccines are similar in both the Friend and Rich viruses.

Cytotoxic Sera

Dr. Lloyd Old and his associates at Sloan-Kettering Institute have described a cytotoxic test system which measures the ability of monospecific mouse sera prepared against a specific leukemogenic virus or leukemic tissue to kill the appropriate leukemic cells when incubated in the presence of complement (*8*).

Using this technique with the Friend, Moloney, Rauscher, and Gross virus leukemias they found that Friend, Moloney, and Rauscher viruses could be placed in one immunological group, which they designated *FMR*, and the Gross virus was placed into a separate group. The specific cytotoxic antisera prepared against any one member of the *FMR* group could be absorbed by any of the other two, but not by the Gross virus. Further, monospecific antisera prepared against Gross leukemia virus or cells had no effect on Friend, Moloney, or Rauscher virus. To determine the position of Rich virus relative to these two distinct antigenic groups, BALB/c mice infected with Rich virus were typed in this cytotoxic system. The results, which were generously provided by Dr. Old, have been reported (*5*). Rich virus reacted equally well against both the *FMR* group and the Gross antigenic group. This suggested that Rich virus, unlike the others listed, shares antigenic components in common with both the *FMR* and Gross virus leukemia groups.

Cell-Specific Antigens

It has been demonstrated in several laboratories that tumor cell transplants in isologous hosts could be inhibited by the prior inoculation of the virus responsible for the initiation of tumor. The specificity of this tumor-specific antigen suggested its use as a method to antigenically distinguish between leukemias induced by viral, physical, or chemical agents (*9*, *10*). Since it had been demonstrated earlier that the leukemia cells induced by our virus could be transplanted in isologous hosts (*4*), this procedure was used to compare the virus isolated in this laboratory with that described by Moloney (*11*).

Groups of 4- to 5-week-old BALB/c mice were inoculated intraperitoneally with undiluted Rich or Moloney virus preparations. Thirteen or 14 days after receiving virus, the mice were inoculated intraperitoneally with the number of viable cells which in control mice would produce readily palpable tumors in 9 to 11 days. With Moloney leukemia cells

TABLE 2.—Effect of prior inoculation of Rich or Moloney virus on the subsequent isologous transplant of leukemic cells induced by these agents

Origin of transplanted cells	Prior inoculation with:	Number of mice with tumor / Number of mice receiving transplant	Incidence* (%)
Rich virus† leukemia	—	8/10	80
"	Rich virus‡	0/10	0
"	Moloney virus‡	6/10	60
Moloney virus† leukemia	—	9/10	90
"	Moloney virus	0/10	0
"	Rich virus	10/10	100

*In experiments with Rich virus leukemia cells, incidence was determined at 11 days after transplantation. Moloney leukemia cells, which had been adapted to an ascites form, grew faster, and incidence of tumor "take" was determined at 9 days.

†The number of viable Rich or Moloney leukemia cells inoculated intraperitoneally was 5×10^6 and 6×10^3, respectively, 13 or 14 days after virus inoculation. Rich leukemia cells were homogenized in glass at 0° C in saline. Whole cells were separated from cellular debris by centrifugation, and viability was determined by trypan blue uptake.

‡BALB/c mice 4 to 5 weeks old received 0.3 ml intraperitoneally of undiluted Rich or Moloney virus preparations.

which had been adapted to a rapidly proliferating ascites form, 6,000 viable cells as determined by trypan blue uptake were employed.

The upper portion of table 2 shows that prior inoculation of Rich virus, but not Moloney virus, resulted in the rejection of a transplant of Rich virus-induced tumor cells; the lower half shows the reciprocal experiment. Rich virus and Moloney virus were tested for their ability to cause rejection of transplanted Moloney virus-induced tumor cells. Prior inoculation of Moloney virus, but not Rich virus, resulted in Moloney cell rejection. These data suggest that those immunological or antigenic determinants responsible for this cell-rejection phenomenon are different in the Moloney and Rich viruses. We did not use this technique for the Friend and Rauscher leukemia viruses because the early splenic response to these viruses obscured the effects of cell transplantation. This could have been overcome by transplantation of cells to a more distant site.

Neutralizing Antisera

Several investigators have attempted to characterize and differentiate the murine leukemia viruses using specific neutralizing antisera prepared in the mouse or rabbit (*7*, *11*, *12*). To date, these attempts have not been completely successful. To continue our study of the relationships among the murine leukemia viruses, experiments with neutralizing antisera were carried out.

The results of initial experiments with a Rauscher virus antiserum made in the rabbit (generously supplied by Dr. Frank Rauscher of the National Cancer Institute) were reported (*5*). Virus was incubated with an equal volume of antisera or normal rabbit serum for 1½ hours at 37° C before inoculation into 4-week-old BALB/c mice. Undiluted anti-Rauscher virus serum neutralized the leukemogenic activity of Rauscher virus. Under the same conditions the anti-Rauscher virus serum did not significantly neutralize an equivalent leukemogenic activity of Rich virus. These experiments were carried out with undiluted sera. To further pursue this aspect of the comparative study, it appeared necessary to use more potent antisera. With a protocol previously reported (*5*), antisera have been prepared to date against Rich virus, Friend virus, and, for control purposes, extracts of normal ICR/Ha Swiss mouse spleen. It should be emphasized that the neutralization studies presented here are not complete, and some data are from experiments still in progress. All of the following neutralization experiments were carried out in ICR/Ha Swiss mice. Table 3 shows the results of an *in vitro* neutralization experiment employing an antiserum prepared against Rich virus. After incubation of the serum for 30 minutes at 56°, virus and varying dilutions of antiserum were incubated for 1 hour at 37° C and the mixtures inoculated into suckling mice. Animals were examined regularly for the development of lymphoma. Results were checked by gross and microscopic pathology. After 71 days all control mice were leukemic (table 3). Fifty % of the mice were protected even when the antiserum had been diluted 1:512. Presentation of the neutralization data in this way is somewhat misleading in that it implies the suggestion of permanent

TABLE 3.—Neutralization of the leukemogenic activity at day 71 of Rich virus by a rabbit antiserum prepared against it

	Antiserum dilution	Number leukemic / Number inoculated*
Rich virus 10^{-1} + normal rabbit serum	1:2†	7/7
Rich virus 10^{-1} + anti-Rich virus serum	1:2	0/7
	1:4	0/6
	1:8	1/8
	1:16	0/7
	1:32	1/7
	1:64	2/8
	1:128	1/8
	1:256	3/8
	1:512	4/8
	1:1024	6/8

---

*The 10^{-1} dilution of virus used to titrate the antiserum contained 5 logs of virus as determined by assay in mice.

†Rich virus at 10^{-1} was incubated (1:1) with the serum that had been heated at 56° C for 30 minutes. These dilutions represent the final dilution of antisera in the virus-serum mixture. Leukemogenic assay was carried out in 1- to 2-day-old Swiss mice (0.05 of the virus-serum mixture intraperitoneally).

Table 4.—Neutralization of the leukemogenic activity at 208 days of Rich virus by a rabbit antiserum prepared against it

Antiserum dilution	Number leukemic / Number inoculated*	Mean latent period (days)	Median latent period (days)
No antiserum†	7/7	65	61
1 : 2	0/7	—	—
1 : 4	2/6	141	—
1 : 8	6/8	128	132
1 : 16	6/7	139	126
1 : 32	7/7	114	108
1 : 64	8/8	93	94
1 : 128	6/6	109	94
1 : 256	7/7	79	80
1 : 1024	8/8	64	51

**See* footnotes table 3.
†Normal rabbit serum 1:2.

protection. This is untrue. Neutralization of murine leukemia viruses, like most other viruses, does not involve a virucidal effect. If this same experiment is observed 208 days after inoculation (table 4), we can see that the protection caused by neutralization is transitory. One can, however, by examination of the *mean* latent period to development of leukemia, or preferably the *median* (leukemia dose 50), the day at which 50% of the animals show leukemia, determine a neutralization endpoint. The endpoints, as determined by early incidence (table 3) and by the method shown in table 4, were essentially equal.

The problem of neutralization endpoints in such a system is somewhat complicated by the factor of age dependence in the animal assay system. If the onset of disease is delayed by the neutralization of virus, the effect is amplified by the decreasing sensitivity of the mouse with increasing age. This will be further considered below.

The leukemia virus (Rich) preparation employed in these studies contained 4 to 6 logs of infectious virus as measured by mouse assay. Thus, these data do not agree with earlier suggestions that leukemia viruses are peculiar in that they lack antigenic potential. Because of the relatively high potency of the antisera, despite the fact that the neutralization was carried out *in vitro*, it was important to determine if nonspecific antimouse activity was in some way responsible for the inhibition of the leukemogenic response. This was determined in several ways.

A potent antiserum against Friend virus was also prepared (table 5). This antiserum was absorbed by incubation with an extract of thymus, spleen, and lymph nodes prepared from normal Swiss mice. In subsequent neutralization experiments, no differences were observed between absorbed and nonabsorbed immune sera in their ability to neutralize the leukemogenic activity of Friend virus. These results suggested a neutralizing activity directed against the virus, rather than the host.

TABLE 5.—Neutralization of the leukemogenic activity of Friend virus by a rabbit antiserum prepared against it

Antiserum dilution	Number leukemic / Number inoculated*	Mean latent period (days)	Median latent period (days)
No antiserum†	5/5	19	21
1:2	0/5	—	—
1:4	1/5	243	—
1:8	0/5	—	—
1:16	1/5	243	—
1:32	0/5	—	—
1:64	2/5	73	—
1:256	4/5	48	37
1:512	4/5	62	26

*Virus treated as in table 3.
†Normal rabbit serum 1:2.

Still further evidence for the virus specificity of the neutralization was obtained by preparing an immune serum against a mixture of spleen, lymph node, and thymus cells of normal Swiss mice, with the same protocol as used for the antiviral sera.

It may be seen that, under conditions in which a 1:64 dilution of antivirus serum neutralized the leukemogenic activity of Rich virus (table 6), a 1:2 dilution of antiserum prepared against normal mouse spleen and thymus cells showed no neutralizing activity. These experiments suggested that the neutralizing effect we had observed was due to an antibody-virus interaction.

It should be pointed out that not every immune serum prepared showed the high potency of the original batch. The serum used in this experiment, for example, neutralized 5 logs of virus, or infectious units, at a dilution of 1:64 in contrast to the 1:256 or 512 seen in earlier experiments. It was, however, sufficiently potent for a comparison of antigenically related viruses. Earliest attempts to determine the cross-

TABLE 6.—Neutralization of the leukemogenic activity (100 days) of Rich virus by normal and immune rabbit sera

	Incidence Number leukemic / Number inoculated*		
Serum dilution	Rich† virus normal rabbit serum	Rich† virus antimouse tissue serum	Rich† virus anti-Rich virus serum
1:2	6/8	4/6	0/7
1:4		6/8	0/6
1:8		6/6	0/7
1:16			0/3
1:32			0/5
1:64			2/7
1:128			5/7

*†*See* footnotes table 3.

TABLE 7.—Neutralization of the leukemogenic activity at 177 days of Rauscher virus by a rabbit antiserum prepared against Friend virus

Antiserum dilution	Number leukemic / Number inoculated*	Mean latent period (days)	Median latent period (days)
No antiserum†	7/7	15	13
1:2	3/8	101	—
1:16	6/8	82	48
1:32	7/8	56	48
1:64	7/8	67	48
1:128	8/8	49	24
1:256	8/8	25	24
1:512	6/7	18	13

*See footnotes table 3. Weanling mice used for assay.
†Normal rabbit serum 1:2.

neutralizing activity of this serum were carried out before the relatively high potency of the immune serum was known (*5*). Cross neutralizations at low dilution in which the virus concentration rather than the serum concentration was varied were done. These experiments, with 1:2 and 1:4 dilutions of immune sera, were not particularly suited for the detection of antigenic differences between these viruses.

Using the immune sera titration data shown in tables 3 through 6, we are presently carrying out neutralization studies in which the immune serum rather than the virus is varied over a wide range of concentrations.

Table 7 shows the results of an experiment in which an immune serum prepared against Friend virus was used to neutralize Rauscher virus: At a dilution of 1:256 it effectively neutralized a 10^{-1} dilution of Rauscher virus. In an experiment not shown, this anti-Friend virus serum neutralized an equal concentration of the homologous Friend virus at the same serum dilution.

In contrast, the immune serum prepared against Rich virus with a homologous titer of 1:64 when reacted with Rauscher virus gave virtually no neutralization (table 8).

TABLE 8.—Neutralization of the leukemogenic activity at 100 days of Rauscher virus by a rabbit antiserum prepared against Rich virus

Antiserum dilution	Number leukemic / Number inoculated*	Mean latent period (days)	Median latent period (days)
No antiserum	6/7	31	29
1:2	3/7	37	—
1:4	4/7	42	29
1:8	5/7	46	29
1:16	4/7	42	29

*See footnotes table 7.

TABLE 9.—Cross neutralization of the leukemogenic activities of Rich and Friend virus by a rabbit antisera prepared against them

Antiserum dilution	Number leukemic / Number inoculated*	Mean latent period (days)	Median latent period (days)
Friend virus			
No antiserum	6/6	15	18
1:32	5/7	48	34
1:64	4/7	32	18
1:128	4/7	43	18
Rich virus			
No antiserum	8/8	98	84
1:32	8/8	123	104
1:64	9/9	120	104
1:128	8/8	106	84

*See footnotes tables 3 and 7.

The approximately equal reciprocal reactions between Friend and Rich virus and their respective immune sera are shown in table 9. Both were effective at an approximate serum dilution of 1: 64.

In table 10, Rich and Friend virus antisera, having approximately the same activity against each other in reciprocal cross neutralizations, show a marked difference in their respective ability to neutralize Rauscher virus. This might be considered in comparing the Friend and Rauscher viruses. It is also of interest that both anti-Friend and anti-Rich virus sera were effective in their neutralizing activity against Moloney virus at a relatively high dilution. The effective neutralization of Moloney virus by the anti-Friend virus serum agrees completely with the earlier published finding by Moloney and Friend on the relationship between these two viruses (*2*, *7*).

In a cross-neutralization experiment it was observed that anti-Rich virus serum neutralized the *homologous* Rich virus at a dilution of 1: 64

TABLE 10.—Cross neutralization of the leukemogenic activities of Rich, Friend, Rauscher, and Moloney virus by the rabbit antisera prepared against Rich and Friend viruses

Antiserum prepared against	Virus	Highest dilution of antiserum yielding effective neutralization
Rich virus	Friend	1: 64
Friend	Rich	1: 64
Rich	Rauscher	1: 2
Friend	Rauscher	1:256
Rich	Moloney	1: 512
Friend	Moloney	1: 1024

(LD50). It was also observed that under the same conditions anti-Rich virus serum neutralized an equivalent concentration (infectious units) of the heterologous Moloney virus at a higher dilution (1:512) than it did the homologous Rich virus. Similarly, an anti-Friend virus serum, which neutralized Friend virus at a dilution of 1:256, neutralized the heterologous Moloney virus at the higher dilution of 1:1024.

Several explanations for this phenomenon might be considered. If we assume that the virus used to prepare the antisera is contaminated with or shares antigenic determinants in common with other murine leukemia viruses, the neutralizing activity of the resultant immune serum would reflect the relative immunological response of the recipient animal, in this instance the rabbit, to the various antigenic components of the virus preparation. The presence of a highly antigenic component either as a contaminating virus or as one of the antigenic determinants of a single viral entity would result in an immune serum particularly effective against that component. The immunological reactivity of this immune serum to a given virus would depend on the relative concentration of that antigen in the virus or virus preparation being tested. More specifically, if all of the murine leukemia viruses shared some highly antigenic component, perhaps associated with their leukemogenic activity, and if, for example, Moloney virus contained more of this component than did Friend virus, one would expect an immune sera prepared against Friend virus to react more strongly with Moloney virus than with the homologous Friend virus.

An alternative explanation for the observed results is in the relative age dependence of these viruses as briefly mentioned above. Since the leukemogenic activity of Moloney virus is more strongly dependent on the age of the recipient host than is the activity of Friend virus, a given degree of virus-antibody interaction, which in turn resulted in a given delay in the availability of active virus to the host, would be reflected to a greater extent in the animals receiving Moloney virus, where even a small increase in host age at the time of contact with virus results in a significant decrease in leukemogenic response. This is not, however, true with Friend virus, where even adult mice can be readily infected. This hypothesis could be tested by the use of viral proliferation rather than the onset of leukemia as the endpoint in neutralization studies. The definitive resolution of these problems must, of course, await extension and repetition of these studies with more purified immunological reagents in a greater variety of host and viral systems.

These data along with other data presented at this Conference indicate that we now possess the techniques enabling us to answer for the murine leukemia viruses those questions which have been answered for other infectious entities—the answers which hopefully will provide insight into the true relationships between these viruses and the diseases they initiate.

We have described the relationships between four leukemogenic systems using several immunological parameters which presumably measure different antigenic determinants. Similarities and differences have been described. Therefore, on choosing between a *family of related viruses* or a *single* leukemia virus, we must, for the present, favor the former. The concept of a single infectious entity is difficult to establish even for viruses about which far more is known.

The advances in our knowledge of the murine leukemias, made since the pioneer work of Ludwik Gross, have depended more on the exploitation of differences than of similarities. In the absence of both sufficient information and, more important, the absence of any immediate need for choosing between a single virus or family of viruses, the hypothesis that encourages and stimulates further investigation would be preferred.

REFERENCES

(*1*) Gross, L.: "Spontaneous" leukemia developing in C3H mice following inoculation, in infancy, with Ak-leukemic extracts, or Ak embryos. Proc Soc Exp Biol Med 76: 27–32, 1951.

(*2*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*3*) Gross, L.: How many different viruses causing leukemia in mice? Acta Haemat (Basel) 32: 44–62, 1964.

(*4*) Rich, M. A., Geldner, J., Johns, L. W., Jr., Kalocsky, M., Meyers, P., Rothstein, E. L., Siegler, R., and Gershon-Cohen, J.: Studies on murine leukemia—nucleic acids. Trans NY Acad Sci 25: 580–589, 1963.

(*5*) Rich, M. A., Geldner, J., and Meyers, P.: Studies on murine leukemia. J Nat Cancer Inst 35: 523–536, 1965.

(*6*) Siegler, R., Geldner, J., and Rich, M. A.: Histogenesis of thymic lymphoma induced by a murine leukemia virus (Rich). Cancer Res 24: 444–459, 1964.

(*7*) Friend, C.: Immunological relationships of a filterable agent causing a leukemia in adult mice. I. The neutralization of infectivity by specific antiserum. J Exp Med 109: 217–228, 1959.

(*8*) Old, L. J., Boyse, E. A., and Stockert, E.: Mouse leukemias. Typing of mouse leukemias by serological methods. Nature (London) 201: 777–779, 1964.

(*9*) Sachs, L.: Transplantability of an X-ray-induced and a virus-induced leukemia in isologous mice inoculated with a leukemia virus. J Nat Cancer Inst 29: 759–764, 1962.

(*10*) Klein, E., and Klein, G.: Antigenic properties of lymphomas induced by the Moloney agent. J Nat Cancer Inst 32: 547–568, 1964.

(*11*) Moloney, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J Nat Cancer Inst 24: 933–951, 1960.

(*12*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

DISCUSSION

Dr. Sinkovics: What is the advantage in diluting the serum and not the virus? We did neutralization tests with virus dilutions, tenfold increasing 5 of them from 10^{-1} to 10^{-5}, against undiluted serum, and we found cross-neutralization from a weak to a moderate degree between Moloney, Friend, and Rauscher viruses; when we used the same sera in the cytotoxic test we found a very strong overlap of cross-reaction.

Dr. Rich: In contrasting the dilution of serum rather than the virus, I was discussing the disadvantages of a system where the antiserum *cannot* be diluted because there is not sufficient neutralizing activity. In searching for a sensitive system to detect similarities and differences between the murine leukemia agents, I would think that neutralizing antiserum should have sufficient activity so that it could be diluted. However, even assuming sufficient activity, there are several reports in the literature (*e.g.*, Fazekas de St. Groth, S., Adv. Virus Res., 9: 1, 1962) citing the advantages of *constant virus-variable serum* versus *constant serum-variable virus* systems for detecting viral neutralization.

Dr. Mayyasi: Dr. Rich, have you done any absorption studies investigating the cross-relationship among these viruses?

Dr. Rich: We have begun some immunodiffusion experiments with absorbed reagents but none as yet with respect to neutralizing activity.

Dr. Reif: Dr. Rich, in your cytotoxic experiments in which you showed an apparent difference between the Rich and Moloney viruses, I think these results would be more meaningful if you were working with Moloney virus isolated from the same strain of mouse as the Rich virus. Otherwise, your virus preparation would be contaminated with mouse strain-specific cellular antigens, and these cellular antigens could give rise to the results you presented. Regarding your last slide, it might have been instructive to have had results on the Gross virus alongside the other data.

Dr. Rich: In reply to the objection raised by Dr. Reif concerning cytotoxicity, the Moloney and Rich viruses and leukemias *were* in the same mouse strain—BALB/c, obtained from the same animal colony. With respect to the use of the Gross virus in our test systems, we could not include all of the available virus isolates, and technical considerations precluded the inclusion of the Gross virus. I am sure other laboratories are carrying out similar studies that include Gross virus.

Dr. Stansly: Dr. Rich has mentioned a number of times that he was under a misconception regarding the antigenicity of the leukemic viruses, being led to believe they were poorly antigenic. I believe lack of antigenicity never referred at any time to heterologous hosts, with which he has been concerned, but rather to the mouse itself, and in this respect it seems to me the Gross virus is still relatively nonantigenic compared to the other leukemia agents.

Dr. Rich: If Dr. Stansly checks the literature pertaining to the antigenicity of murine leukemia viruses, he will find that I have misinterpreted neither the data reported nor direct statements as to the interpretation of these data. He will find in the literature specific suggestions that the poor antigenicity (heterologous) observed has inhibited the immunological differentiation of the murine leukemia viruses [*e.g.*, Gross, L., Acta Haemat (Basel) 32: 44–62, 1964]. With regard to another point, Dr. Gross mentioned earlier that the same antigenic differences seen in Rous, polyoma, influenza, and polio viruses must also exist for the murine leukemia viruses. I could not agree more with this concept. These are the differences that make up families of viruses, in the tumor viruses as well as in the nontumor viruses. There is no reason to say definitively at this time that there is one virus or several viruses. What we really have to do is to elucidate the mechanisms by which these viruses cause leukemia.

Dr. Klein: I would like to congratulate Dr. Rich for a beautiful piece of work and clear presentation. May I ask one question regarding the cross-resistance test between Rich and Moloney viruses? As you showed, there was no cross-reactivity in the transplantation test, and yet in the test for absorption of cytotoxic activity from immune sera you found cross-reactivity. Did you test the sera of animals resistant against Rich and Moloney viruses, respectively, against the corresponding target cells in the direct cytotoxic test?

Dr. Rich: Thank you, Dr. Klein. In reply to your question we have not as yet tested these sera.

Dr. Mora: I would like to point out that we have now reached that level in techniques when one can compare the various mouse leukemia viruses by extracting intact nucleic acid, similar to our method presented on the Rauscher virus. By purifying other mouse leukemia viruses, and comparing the isolated intact nucleic acids, one would have a criterion, in addition to immunological methods, pertaining to the viruses but without the complicating factors of the outside virus coat which is of host cell origin. Base ratio analyses now are being carried out on the Rauscher RNA extract in our laboratory.

A Comparison by Several Techniques of the Antibody Prepared in Various Species Against the Rauscher Murine Leukemia Virus [1]

Mary Alexander Fink, Carolyn A. Cowles, Michael A. Chirigos, *and* J. Messore, *Laboratory of Viral Oncology, National Cancer Institute,*[2] *Bethesda, Maryland*

In the last decade various attempts have been made to determine the antigenicity of murine leukemic viruses, and the relationships among the strains isolated by different investigators (*1*). Results and interpretations of these tests have sometimes differed from laboratory to laboratory, possibly depending on the variation in the species and strain of animal in which antiserums were produced, and the type of immunological study made. Most of our previously reported work was done in a completely isologous system, in which active and passive immunization and neutralization techniques demonstrated the presence of a protective antibody against Rauscher virus in immunized mice of the BALB/c strain, in which this virus was discovered and serially propagated (*2, 3*). The need for potent, specific antiserums in relatively large quantity has prompted the use of several larger rodent species and one primate species for the production of antibody. The results of *in vivo* and *in vitro* tests of these various antiserums are reported herein.

MATERIALS AND METHODS

Preparation of serums.—The general protocol for the preparation of serums was as follows: *Heterologous serums*—adult Rhesus monkeys, Hartley strain guinea pigs, Race III rabbits, and Osborne-Mendel rats received a primary intraperitoneal inoculation of 2 ml. This was composed of 1 ml 10 times concentrated plasma, from Rauscher virus-infected BALB/c mice, thoroughly emulsified in an equal amount of complete

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Freund's adjuvant. A minimum of 3 weeks later these animals were given a subcutaneous booster inoculation of 0.10 to 0.25 ml without adjuvant. They were bled 7 to 10 days later and the serums harvested. After inactivation at 56° for 30 minutes, these serums were tested for reactivity with sheep erythrocytes, BALB/c erythrocytes, and normal BALB/c plasma. Serums were absorbed with these materials until no reaction was demonstrable by hemagglutination in the case of the cells and double diffusion in the case of the plasma. *Homologous serum* was prepared by the inoculation of a formalin-treated 10% cell-free extract of leukemic spleen plus adjuvant. Many details of these experiments have been previously reported (*2*, *3*).

In vivo tests of serums.—Immune serums prepared in the monkey, rabbit, guinea pig, and mouse were tested by these techniques. *Neutralization tests* were performed by two techniques previously described (*2*, *3*). Each of these was performed by mixing various virus dilutions with antiserum, incubating the mixtures at room temperatures, and then inoculating them into BALB/c mice. The duration of the longer test was 120 days, and neutralization was judged by reduction in splenomegaly or death from leukemia by 120 days. In the second, shorter method the spleen-focus assay was utilized and the endpoint was the reduction of the number of spleen colonies as compared to controls after 7 or 8 days' incubation. *Passive immunization* techniques included the intraperitoneal administration of inactivated, absorbed heterologous serums and homologous serums in 0.5 to 1 ml quantities approximately 20 hours before the inoculation of live virus, as reported previously (*1*). Tests to determine a therapeutic effect of antiserum in the presence of viremia were carried out in BALB/c mice using the protocols described by Chirigos (*4*) and briefly mentioned in a previous publication (*2*). The results presented here were based on the administration of 0.25 to 0.3 ml of antiserum on 3 and on 6 days after the inoculation of virus. Seven days after the viral inoculation, the amount of virus present in the plasma was determined by bioassay in recipient mice.

In vitro test.—The *in vitro* testing of antiserums was confined to the performance of a micro-double-diffusion test. Preliminary experiments indicated that the microtechnique of Wadsworth and Crowle, using 0.85 to 1% ionagar as the medium for use in double-diffusion studies, was applicable to tests with the murine leukemia viruses (*4*). In brief, the reactant wells were filled and the preparations incubated from 48 to 72 hours at room temperature, after which they were examined and permanent photographic records made of the reactions. Since the preliminary studies, it has been found that 0.75% Agarose[3] allows more rapid diffusion of the reactants and is a less friable medium to work with.

Each of the heterologous serums, after complete absorption, was tested against Rauscher virus preparations from various sources and gave specific reactions. The plasma of infected mice was used as test antigen, either unconcentrated or concentrated in the case of the Rauscher virus, 10 times concentrated Friend virus, and 100 times concentrated Moloney virus. An additional control was use of the plasma of BALB/c mice that had been inoculated with the third passage of Riley's agent [lactic dehydrogenase (LDH)].

---

[3] SeaKem Agarose, manufactured by Marine Colloid, Inc., distributed by Bausch and Lomb. We are indebted to Dr. E. Leise for suggesting the use of this gel.

To determine the reliability of a scheme devised to differentiate between Rauscher and Moloney viruses using the micro-Ouchterlony technique, a series of 6 coded preparations, known to be Rauscher virus, Moloney virus, or normal control material, was prepared for us by Dr. Erling Jensen of the Charles Pfizer Co. The source of these antigens is indicated in table 3.

All tests were done at least in duplicate on two separate occasions.

RESULTS

The data in table 1 show the results of neutralization tests, passive protection tests, and tests for a therapeutic effect as evidenced by a reduction of the existent viremia. Each of the immune serums contained protective antibody measurable by some of these techniques, whereas normal serum from the same species did not. When repeated runs on one serum by the spleen-focus assay reduction method of neutralization gave highly variable results, the test was considered unreliable for this purpose and the results have been omitted from the table. It was seen in previous studies that a homologous antiserum had a strong passive protective effect when administered before challenge. Similarly, but to a less extent, these results show that another rodent antiserum, that of the rabbit, showed the effect whereas the monkey antiserum, also with a high neutralizing titer, did not. The results of therapeutic testing in general agree with those measuring the neutralizing antibody.

Table 2 summarizes the results of the Ouchterlony testing of the Rauscher, Friend, and Moloney viruses. It can be seen that only the monkey

TABLE 1.—Protective antibody against Rauscher virus infection demonstrable by several techniques in serum from several species

Species	Status	Neutralization index (log)	Passive protection	Therapeutic reduction of viremia (log)
Mouse	Normal	Negative	Negative	Negative
	Immune	>3.5	Excellent	3.1
Guinea pig	Normal	Negative	Not done	Negative
	Immune	>5.0	Not done	3.2
Rabbit	Normal	Negative	Negative	Negative
	Immune	2.6	Fair	2.6
Monkey	Normal	Negative	Negative	Negative
	Immune	>2.6	Negative	2.4

TABLE 2.—Results of Ouchterlony tests with anti-Rauscher virus serums prepared in various species against Rauscher, Friend, and Moloney virus from the plasma of infected BALB/c mice

Immune serum	Minimal number of lines of reactivity with:		
	Rauscher	Friend	Moloney
BALB/c mouse	2–3	1–2	0
Guinea pig	3–4	1–2	0
Rabbit	2	1	0
Rat	1	1	0
Monkey	3–4	2	1

serum reacted with Moloney virus, whereas the mouse, monkey, guinea pig, and rabbit serums all reacted with both the Rauscher and the Friend virus but not with the Moloney virus. The one antigen present in the Moloney virus which reacted with monkey antiserum, cross-reacted with, but was not necessarily identical to, one of the lines present in both Rauscher and Friend. These results are graphically presented in figure 1. No reaction occurred with any serum when concentrated LDH was used as the antigen.

Based on these findings—that only the monkey antiserum reacted with Moloney virus, while the BALB/c mouse and guinea pig serum reacted with Rauscher virus but not Moloney virus—a series of unknowns were tested, and the results of these tests are shown in table 3. The accuracy of the judgment made on the basis of these reactions and the sensitivity of the test in detecting viral particles reflect the potential usefulness of this type of test for the detection of murine leukemia viruses.

DISCUSSION

The positive results obtained with the various antiserums indicate the reliability of a general protocol of immunization which includes the use of only two inoculations, the primary one by the intraperitoneal route with Freund's adjuvant, and the second without adjuvant after a minimal interval of 3 weeks. This protocol, used previously for many years to provide highly potent antiserums against various antigens other than murine leukemia, is thus applicable to the preparation of potent antiserums against a murine leukemia virus in various species.

In assaying these antiserums for a protective effect against viral infection, three tests were run. These included neutralization tests in which virus was mixed with antiserum prior to introduction into an animal; therapeutic tests in which attempts were made to reduce the amount of virus in an existent viremia; and passive protection tests in which antibody was administered several hours before the administration of virus. Isologous mouse antiserum protected in any of these instances and had a

Table 3.—Results of testing by Ouchterlony technique of unknown samples* of either Rauscher or Moloney viruses or control materials

Jensen code No.	Reaction with antiserum prepared in:			Conclusion regarding identification	Actual composition of unknown	Particle count by electron microscopy
	Mouse (BALB/c)	Guinea pig	Monkey			
1	Negative	Negative	1 line	Moloney	Moloney JLSV-8† (1,000×)	TNTC‡
2	1 line	1 line	2 lines	Rauscher	Rauscher JLSV-5 high infectivity	100–500
3	1 or 2 lines	2 lines	2 lines	Rauscher	Rauscher JLSV-5 low infectivity	TNTC
4	Negative	Negative	Negative	Normal	JLSV-6 uninoculated	100–500
5	Negative	Negative	1 line	Moloney	Moloney mouse plasma (100×)	50–100
6	1 or 2 lines	3 lines	3–4 lines	Rauscher	Rauscher mouse plasma (10×)	TNTC

*We are indebted to Dr. E. Jensen of the John L. Smith Memorial for Cancer Research, Charles Pfizer Co., for the preparation of these samples.

†JLSV-6 is a mixture of BALB/c mouse embryo spleen and thymus cells established and maintained in tissue cultures at the Charles Pfizer Co. Laboratories. This cell strain was recently observed to be carrying an unidentified viral particle. When the culture is inoculated with Rauscher virus, it is called JLSV-5; with Moloney virus, JLSV-8.

‡TNTC=Too numerous to count.

great effect as measured by passive protection. Other rodent antiserums also protected under these circumstances. The monkey antiserum, potent as judged by neutralization and the reduction of viremia, failed, however, to protect passively. This could be due to a physical incompatibility of the primate gamma globulin molecule that resulted in failure of attachment to appropriate sites on mouse cells.

The *in vitro* studies in which the Ouchterlony technique was used to detect the antigens of the Rauscher virus and the presence of cross-reacting antigens of the Friend and Moloney viruses, as detected by the use of antiserum made against Rauscher virus, present one puzzling finding. A very strong antiserum, as judged by protective antibody content and by its reactivity in double diffusion tests with the Rauscher virus, was the guinea pig serum. But this serum failed repeatedly to react with Moloney virus preparations. Similarly, rabbit antiserum and the homologous BALB/c antiserum also failed to react with Moloney virus. Only the monkey antiserum reacted with the Moloney virus, detecting one line of reactivity which cross-reacted with one line of the Rauscher and Friend viruses. One possible and obvious explanation for this finding—not necessarily the correct one—but one which must be ruled in or out is that the virus against which the monkey antiserum was made was, in effect, a mixture of Moloney and Rauscher viruses. The antigens used in the preparation of all antiserums, except the monkey, were obtained from early stocks prepared by Dr. Rauscher, in whose laboratory great care had been taken to exclude the presence of any other known murine leukemia viruses. The antigen used in the preparation of the monkey antiserum, however, was from a source where both Moloney and Rauscher viruses were prepared in close proximity. The presence of Moloney virus in the Rauscher preparation would not be detected by electron microscopy and probably not detected by infectivity studies, since the rapidly lethal Rauscher virus would tend to obscure any effect of the slowly acting Moloney virus. If certain of the murine leukemia virus stocks have been contaminated, this might explain the disparity of results coming from different laboratories on the relationships among the Moloney, Rauscher, and Friend viruses.

The Ouchterlony test as applied to murine leukemias as in a preliminary stuly been exploited to detect the presence of either Rauscher or Moloney viruses in a series of unknowns. The coded unknowns sent by Dr. Jensen to this laboratory were accurately identified by use of the reaction with guinea pig, BALB/c mouse, and monkey antiserum. The 6 preparations included 3 of Rauscher virus—a high-particle-count plasma virus and 2 preparations from tissue culture; 2 of Moloney virus—a high-particle-count tissue culture virus and a plasma-derived virus; and 1 from the same tissue culture cells, known to be producing a virus, but uninoculated with any murine leukemia virus. These findings, together with the preliminary results showing that Rauscher virus can be detected without concentration in the plasma of infected mice (*5*), suggest a technique which will likely provide the most definitive results on antigenic relationships of the murine leukemia viruses.

SUMMARY

Antiserums against the Rauscher murine leukemia virus were prepared in the isologous host, the BALB/c mouse, and in Rhesus monkeys, rabbits, and guinea pigs. The presence of protective antibody was determined by neutralization tests, passive immunizatiton, and tests designed to detect any reduction of viremia in a previously infected animal. Double diffusion studies were carried out *in vitro* to determine the reactivity of each serum with Rauscher virus. This test was used to test for some cross-reacting antigens of the Friend, Moloney, and Rauscher leukemia viruses. A practical application of this Ouchterlony technique is not only to detect Rauscher virus, but to differentiate between Rauscher and Moloney viruses when these two are presented as unknowns.

REFERENCES

(*1*) Old, L. J., and Boyse, E. A.: Antigens of tumors and leukemias induced by viruses. Fed Proc 24: 1009–1017, 1965.

(*2*) Fink, M. A., and Rauscher, F. J.: Immune reactions to a murine leukemia virus. I. Induction of immunity to infection with virus in the natural host. J Nat Cancer Inst 32: 1075–1082, 1964.

(*3*) Fink, M. A., Rauscher, F. J., and Chirigos, M.: Some immune reactions of murine leukemia viruses demonstrable within a completely isologous system. *In* Viruses Inducing Cancer—Implications for Therapy (Burdette, W. J., ed.). Univ Utah Press, 1966, pp 25–50.

(*4*) Chirigos, M.: Studies with the murine leukemogenic Rauscher virus. III. An *in vivo* assay for anti-viral agents. Cancer Res 24: 1035–1041, 1964.

(*5*) Fink, M. A., and Cowles, C. A.: Detection of a murine leukemia virus (Rauscher) by immunodiffusion. Science 150: 1723–1725, 1965.

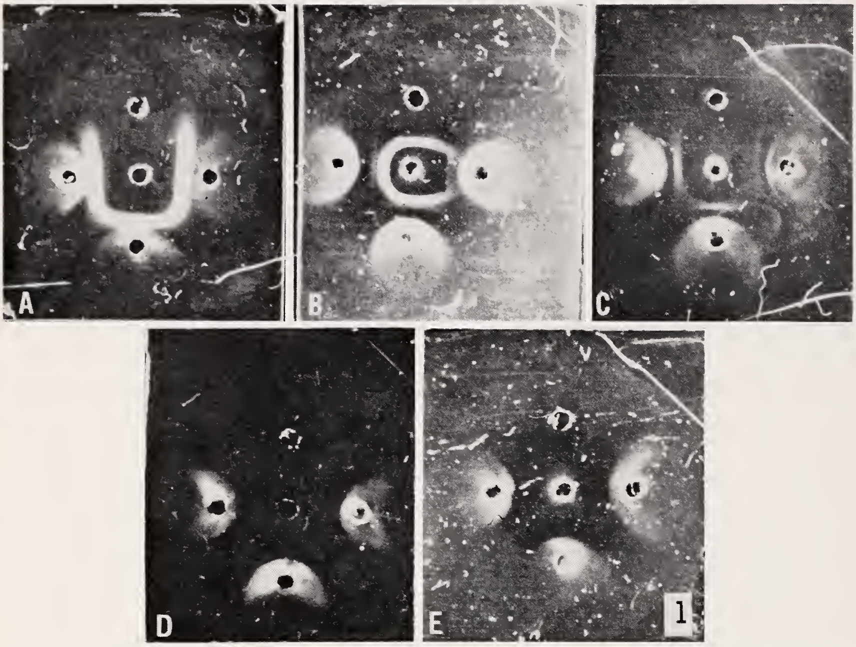


Figure 1.—Results of Ouchterlony tests on murine leukemia viruses with antiserums prepared in various species.

	9 O'clock	12 O'clock	3 O'clock	6 O'clock	Center well
A	RP 10×	MP 100×	FP 10×	RP 10×	BALB/c anti-R
B	"	"	"	"	Monkey anti-R
C	"	"	"	"	Guinea pig anti-R
D	"	"	"	"	Rabbit anti-R
E	"	"	"	"	Rat anti-R

RP—Rauscher plasma virus
MP—Moloney plasma virus
FP—Friend plasma virus

DISCUSSION

Dr. Wright: I would like to ask Dr. Fink if she got any kind of a precipitant line using the V6 antibody with control material—not checking it against the Friend, Rauscher, or Moloney, but checking it against the V6 antigen itself.

Dr. Fink: The only antiserum that we used is against the Rauscher virus. This reacts with JLSV-5, the Rauscher virus prepared in the JLSV-6 cell, but not with JLSV-6 control antigen. We are making, but have not yet tested, an antiserum that is made against the JLSV-6.

Addendum in proof:

Monkey antiserum to Rauscher virus prepared using an early virus isolate from Dr. Rauscher's laboratory also reacted with Moloney virus.

Serological Analysis of Leukemia Antigens of the Mouse[1, 2]

Tadao Aoki, Lloyd J. Old, *and* Edward A. Boyse, *Division of Immunology, Sloan-Kettering Institute for Cancer Research, Sloan-Kettering Division, Cornell Medical College, New York, New York.*

During the past few years, the serological investigation of leukemias in inbred mice has made it possible to classify them in several distinct antigenic types (*see 1*). The methods that have been of greatest value are the cytotoxic test and, later, the immunofluorescence technique, with antisera prepared in the mouse and tested with viable leukemia cells. Five antigenic types have been distinguished by these methods.

The G (Gross) antigen, first recognized by Slettenmark-Wahren and Klein (*2*) in leukemias induced by Passage A Gross virus, is now known to be present in leukemia cells and also in normal lymphoid cells of mice from strains that have a high incidence of spontaneous leukemia—AKR, AKR.K, C58, F, PL, and C3Hf/FgLw; these strains (*see* list) are referred to as G+ strains (*3*).

Occurrence of G (Gross) antigen

All leukemias induced by Passage A Gross virus
All leukemias of AKR, C58, and other strains with high incidence of leukemia
Normal lymphoid tissues of all mice from strains with high incidence of leukemia (AKR, AKR.K, C58, F, PL, C3Hf/FgLw)
Some leukemias and solid tumors of low-incidence strains

The designation FMR refers to a group of cross-reacting antigens found in the cells of leukemias induced by the Friend, Moloney, and Rauscher viruses.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by Public Health Service grant CA–06338, contract PH 43–65–99 from the National Cancer Institute, and a grant from the John A. Hartford Foundation, Inc.

FMR (Friend, Moloney, Rauscher) antigens

Cross-reacting system of antigens in cells of leukemias induced by Friend, Moloney, and Rauscher viruses
Not found in spontaneous leukemias or in leukemias induced by radiation or chemical carcinogens

By the cytotoxic technique, the antigens of Friend and Rauscher leukemia cells appear identical (*4*). The close relationship of the Moloney leukemia antigen is shown by absorption analysis in which antibody produced against any one of the three FMR leukemia types can be completely absorbed by any one of the three (*5*). The most prominent features distinguishing the FMR group from the G system are: 1) The absence of FMR antigens from the lymphoid tissues and leukemias of all mice not experimentally infected with viruses of the FMR group (in contrast to G antigen, which appears naturally in the normal tissues and spontaneous leukemias of some mice), and 2) FMR antibody is not absorbed by G+ leukemias.

The G and FMR antigens are related to identified leukemogenic viruses whereas the third antigenic system, ML, is related to the virus that causes mammary tumors in mice, hence the designation ML (mammary leukemia) antigen (*see* list) (*6*).

Occurrence of ML (mammary leukemia) antigen

Spontaneous leukemias of DBA/2 mice
Spontaneous mammary tumors of MTV+ mice
Normal mammary tissue of MTV+ mice

This antigen has been found in the leukemias of only DBA/2 mice, but it occurs also in the mammary tissues and mammary tumors of all mice infected with the mammary tumor virus (MTV). The relationship of ML antigen to the MTV has been established by studies with reciprocal crosses and foster-nursed mice, in which the presence of ML antigen invariably followed the maternal transmission of MTV. The explanation best accounting for the occurrence of ML+ leukemia in DBA/2 mice is that this strain has a variant of MTV with leukemogenic properties (*1*).

Very little is yet known about the fourth antigen, E, except that it appears to be restricted to the leukemias of one strain, C57BL, and is not associated with any identified virus (*1*).

The fifth system, TL (thymus leukemia), represents a class of antigens that so far appears unique in tumor immunology (*7*):

Properties of TL (thymus leukemia) antigen

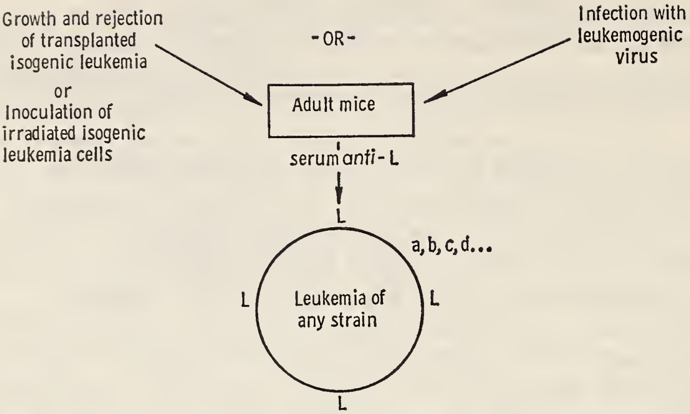
Organ-specific isoantigen	Present in normal thymus cells of certain strains of mice (TL+ strains) Determined by a dominant gene adjacent to *H-2* in linkage group IX
Leukemia-specific antigen	Occurs in leukemias of mice that lack TL antigen (TL− strains)

It is the only example of a leukemia-specific antigen that is coded by the genome of the cell. In mice of certain strains (TL+ strains) TL antigen is present in normal thymus cells, and its appearance in leukemias of these strains indicates that these cells have arisen from thymus cells. In TL− strains, which do not possess TL antigen in any normal tissue at any time of life and are capable of forming TL antibody, leukemias frequently have TL antigen. The presence of TL antigen in leukemias of TL− strains, where it is clearly a leukemia-specific antigen, has been explained as release of the TL structural gene from a control mechanism that normally suppresses formation of TL antigen in TL− strains.

These systems were defined originally by the cytotoxic test of Gorer and O'Gorman (*8*, *9*). In this test, counted numbers of viable cells are incubated with serial dilutions of mouse antiserum; since mouse serum does not complement the reaction, guinea pig serum is added. After 45 minutes, the cells are counted in the presence of trypan blue, which stains dead cells. The dilution of serum in which approximately 50% of the cells are stained is taken as the endpoint. The use of absorption greatly extends the range of the test in the study of leukemia-specific antigens. Cells in which the content of a particular antigen is low may show little or no sensitivity in the direct cytotoxic test, and yet may show strong specific absorption of cytotoxic antibody. Maximal sensitivity is achieved by selection of the most susceptible cell as the standard and dilution of the typing serum, before absorption with the tissue to be tested, in accordance with the endpoint of the typing serum against the standard test cell.

The most important step in the analysis of any of these systems is the preparation of an adequate typing serum. Several methods have been used for preparing antisera [reviewed in (*1*) and (*10*)]. Ideally, the antiserum should be prepared in such a way that no isoantibodies are formed as a result of immunization. Antisera of this type can be prepared by immunizing adult mice with: 1) viable isogenic leukemia cells, so highly antigenic that the grafts regress, 2) irradiated isogenic leukemia cells, or 3) virus (text-fig. 1). As virus is released from transplanted leukemia cells, the immunizing stimulus may in all cases be brought about mainly by the limited replication of virus in host cells. In the text-figure, isoantigens are represented by the small letters *a*, *b*, *c*, etc.; antibodies against these antigens cannot be formed in this type of immunization.

In those instances where the host is immunologically tolerant of the antigen under investigation, as, for example, in the case of G antigen in AKR (G+) mice or TL antigen in TL+ mice, it is not possible to produce antiserum in the strain of origin; under these circumstances, isoimmunization of foreign strains must be employed (text-fig. 2). Isoantigens are again represented here by the small letters *a*, *b*, etc., and in this case the corresponding antibodies are formed. The leukemia-specific antigen is represented by the capital letter *L*. With sera of this sort, the test must be so designed that the participation of isoantibodies is excluded. This may be done in two ways. The antiserum may be used only with leukemias

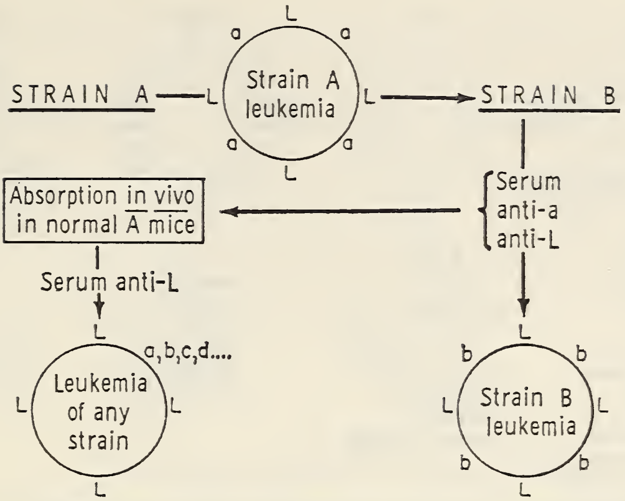

Growth and rejection of transplanted isogenic leukemia
or
Inoculation of irradiated isogenic leukemia cells
-OR-
Infection with leukemogenic virus
Adult mice
serum anti-L
L
a,b,c,d...
L
Leukemia of any strain
L
L

Text-figure 1.—Detection of specific leukemia antigen by immunization with virus or with isogenic leukemia.

arising in the strain in which the antiserum was produced. Alternatively, the isoantibodies may be removed by absorption with normal tissues, and theoretically this can be performed either *in vitro* or *in vivo.* Absorption *in vitro* has the limitation that it generally must be repeated several times with large amounts of normal tissue, and even so, the tissues used for absorption may not contain a sufficient amount of all isoantigens to ensure complete absorption from undiluted serum. For this reason, the technique of absorption *in vivo*, described by Amos (*11*), has considerable advantage over absorption *in vitro.* The antiserum to be absorbed is injected into normal mice of the same genotype as that of the leukemia used for immunization and is recovered 4 hours later by cardiac puncture. The completeness of absorption is indicated by negative cytotoxic tests with normal lymphoid cells and by failure of normal tissue to absorb the residual specific cytotoxic antibody. No matter which of these methods is used for obtaining a typing reagent, the presence of antigen in tissue of *any* genotype can be determined by absorption of the suitably diluted specific antiserum with the tissue to be examined, in tests with a selected sensitive typing leukemia cell.

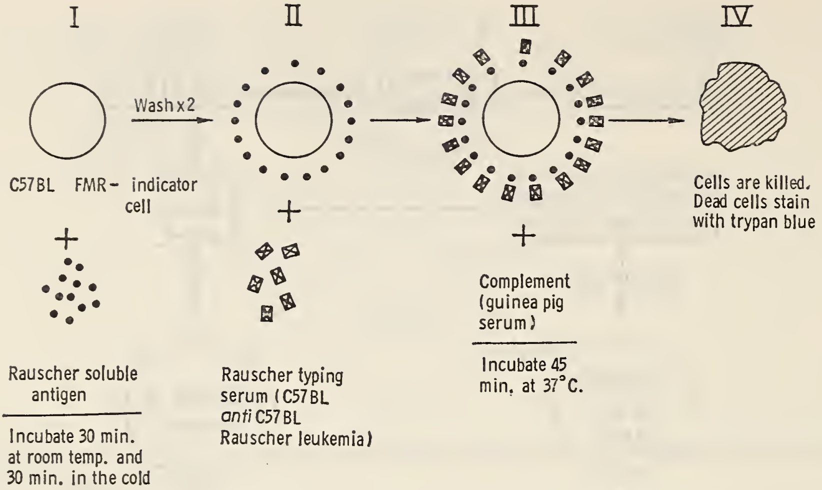
Of the typing sera available to us, the G and TL have been produced by isoimmunization of C57BL/6 mice with G+ or TL+ leukemias and are used in tests with C57BL/6 G+ or TL+ leukemias. The ML and E systems were defined with isoimmune sera absorbed *in vivo.* In general, the FMR system was analyzed with the serum of mice that had rejected transplants of isogenic leukemias that were highly antigenic in the home strain. In most instances, prolonged courses of immunization are necessary for the production of satisfactory typing reagents.

We have so far discussed the use of the cytotoxic technique only in relation to the detection of the specific antigens present on leukemic cells. These tests are performed with viable cells and the presumption is that



Text-figure 2.—Detection of specific leukemia antigen by isoimmunization.

the antigens constitute part of the cell membrane and may represent viral protein. In the FMR system, a number of sera active in the cytotoxic and immunofluorescence tests have been shown to have virus-neutralizing activity (*12*, *13*), although it is not known that this antibody has the same specificity. In addition to antigen demonstrable in infective virus and on the viable leukemia cells, a third class of antigen has recently been found in leukemic mice (*12*). These have been termed soluble because they are not sedimented by centrifugation sufficient to remove infective virus. The soluble antigens of mouse leukemias were first observed as a consequence of experiments concerned with the ability of leukemia viruses to infect the cells of transplanted leukemias of unrelated antigenic types. These experiments showed that Rauscher virus could permanently infect EL4 cells and the cells of other transplanted leukemias that do not contain Rauscher leukemia antigen, and that these cells now produce Rauscher antigen (*14*). This is the phenomenon called "antigenic conversion." Further experiments were carried out to see if adsorption of virus alone, as distinct from established infection, would render cells sensitive to Rauscher antiserum. In these tests C57BL ascites EL4 leukemia cells, which are FMR−, were exposed for 60 minutes to infective BALB/c plasma (the usual source of infective Rauscher virus) and the cells were now found to be sensitized to Rauscher antiserum in the cytotoxic test (text-fig. 3.) Infective BALB/c plasma was then centrifuged at 100,000 × g for 1 hour, and the pellet and supernatant were tested both for infectivity and for the presence of antigen capable of sensitizing EL4 cells to Rauscher antiserum. As expected, infectivity was confined to the pellet. Sensitizing antigen, however, remained predominantly in the supernatant. For this reason, this antigen was called soluble. Soluble

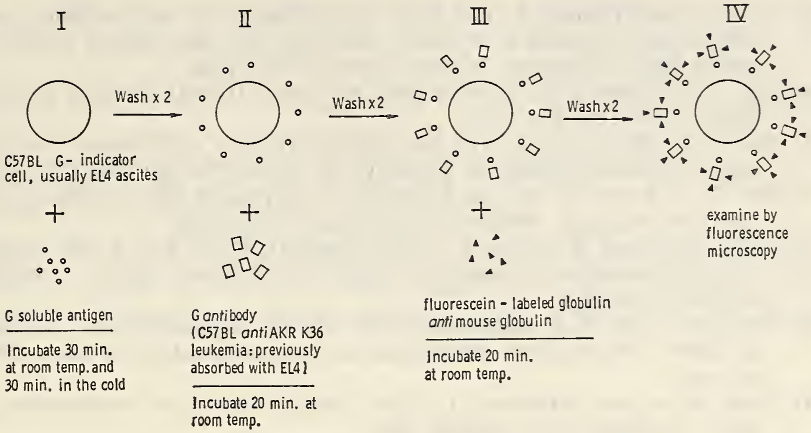

I
II
III
IV
Wash x2
C57BL FMR- indicator cell
+
Rauscher soluble antigen
Incubate 30 min. at room temp. and 30 min. in the cold
+
Rauscher typing serum (C57BL *anti* C57BL Rauscher leukemia)
+
Complement (guinea pig serum)
Incubate 45 min. at 37°C.
Cells are killed. Dead cells stain with trypan blue

TEXT-FIGURE 3.—Detection of soluble antigens of the FMR group.

antigens with the property of adsorption to viable indicator cells with consequent sensitization to specific antisera have been detected also in the plasma of mice with Friend and Moloney leukemias. As with the cytotoxic test on Friend, Moloney, and Rauscher leukemia cells, cross-reactions are observed with soluble antigens adsorbed on indicator cells. Once again in conformity with the result obtained by cytotoxic tests with viable cells, FMR soluble antigens were not found in the plasma of normal mice, nor in the plasma of mice with spontaneous leukemias or leukemias induced by any means other than the inoculation of the Friend, Moloney, and Rauscher viruses (*12*).

It was not possible to detect a soluble antigen with G specificity by similar means in the plasma of mice with leukemias induced by Passage A Gross virus or with spontaneous G+ leukemias. We then considered the possibility that G soluble antigen might be present, but that the quantities adsorbed were not sufficient to sensitize the carrier cell to the cytotoxic reaction of G antibody and complement. For this reason, we turned to the indirect immunofluorescence method applicable to viable cells (*15*) as an alternative way of demonstrating antigen adsorbed on the cell surface. This led to the demonstration of soluble G antigen, and we shall now give a brief account of our studies of G soluble antigen. Text-figure 4 illustrates the technique. The G− indicator cell is again the C57BL ascites leukemia EL4. Other C57BL leukemias may be used. Counted numbers of cells are incubated first with the plasma or other material to be tested for G soluble antigen, then with G antibody, and finally with fluorescein-labeled rabbit (*anti*-mouse globulin) globulin. The presence of adsorbed G antigen is indicated by discontinuous fluorescence of the cell membrane.

From the results obtained *in vitro* with soluble antigens adsorbed onto viable cells, it is not surprising to find that large amounts of soluble antigen


I
II
III
IV
Wash x 2
Wash x2
Wash x2
C57BL G- indicator cell, usually EL4 ascites
+
+
+
examine by fluorescence microscopy
G soluble antigen
Incubate 30 min. at room temp. and 30 min. in the cold
G *anti*body (C57BL *anti*AKR K36 leukemia: previously absorbed with EL4)
Incubate 20 min. at room temp.
fluorescein - labeled globulin *anti* mouse globulin
Incubate 20 min. at room temp.

Text-figure 4.—Detection of G soluble antigen.

are adsorbed *in vivo* onto the cells of mice infected with these viruses and onto the leukemia cells themselves. By the gentle use of a loose-fitting hand-homogenizer, insufficient to cause considerable disruption of cells, it is possible to separate soluble antigen of either G or FMR specificities from suspensions of leukemia cells in concentrations as high as those in plasma, and often much higher. If the cells were previously washed, soluble antigen is obtained in a relatively pure state. Thus the soluble antigen that is found free in the plasma is probably only a small fraction of the total soluble antigen present in the host.

The pattern of distribution of G soluble plasma antigen in mouse populations follows precisely the occurrence of G cellular antigen. All mice of all strains with a high incidence of spontaneous leukemia have both G soluble plasma antigen and G cellular antigen throughout life. The test for soluble G antigen in the plasma thus permits the unequivocal identification of mice from strains with high incidence of leukemia, long before they develop leukemia. The mice may then be retained for repeated sampling and for further observation. The invariable finding of G soluble plasma antigen in G+ mice provides an index of the presence of naturally occurring leukemia virus and will greatly facilitate analysis of the transmission of virus in the segregating progeny of crosses between G+ and G− strains. We shall then begin to know more about the spread of leukemia viruses in natural populations.

REFERENCES

(*1*) Old, L. J., and Boyse, E. A.: Antigens of tumors and leukemias induced by viruses. *In* Symposium on Immunopathology of Cancer. Fed Proc 24 (I): 1009–1017, 1965.

(*2*) Slettenmark-Wahren, B., and Klein, E.: Cytotoxic and neutralization tests with serum and lymph node cells of isologous mice with induced resistance against Gross lymphomas. Cancer Res 22: 947–954, 1962.

(*3*) Old, L. J., Boyse, E. A., and Stockert, E.: The G (Gross) leukemia antigen. Cancer Res 25: 813–819, 1965.

(*4*) Old, L. J., Boyse, E. A., and Lilly, F.: Formation of cytotoxic antibody against leukemias induced by Friend virus. Cancer Res 23: 1063–1068, 1963.

(*5*) Old, L. J., Boyse, E. A., and Stockert, E.: Typing of mouse leukemias by serological methods. Nature (London) 201: 777–779, 1964.

(*6*) Stück, B., Boyse, E. A., Old, L. J., and Carswell, E. A.: ML—A new antigen found in leukemias and mammary tumours of the mouse. Nature (London) 203: 1033–1034, 1964.

(*7*) Boyse, E. A., Old, L. J., and Stockert, E.: The TL (thymus leukemia) antigen. A review. International Symposium on Immunopathology, Monaco, 1965. In press.

(*8*) Gorer, P. A., and O'Gorman, P.: The cytotoxic activity of isoantibodies in mice. Transplant Bull 3: 142–143, 1956.

(*9*) Boyse, E. A., Old, L. J., and Stockert, E.: Some further data on cytotoxic isoantibodies in the mouse. Ann NY Acad Sci 99: 574–587, 1962.

(*10*) Old, L. J., and Boyse, E. A.: Immunology of experimental tumors. Ann Rev Med 15: 167–186, 1964.

(*11*) Amos, D. B.: The persistence of mouse iso-antibodies *in vivo*. Brit J Cancer 9: 216–221, 1955.

(*12*) Stück, B., Old, L. J., and Boyse, E. A.: Occurrence of soluble antigen in the plasma of mice with virus-induced leukemia. Proc Nat Acad Sci 52: 950–958, 1964.

(*13*) Klein, E., and Klein, G.: Mouse antibody production test for the assay of the Moloney virus. Nature (London) 204: 339–342, 1964.

(*14*) Stück, B., Old, L. J., and Boyse, E. A.: Antigenic conversion of established leukemias by an unrelated leukaemogenic virus. Nature (London) 202: 1016–1018, 1964.

(*15*) Möller, G.: Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. J Exp Med 114: 415–434, 1961.

DISCUSSION

Dr. Sinkovics: Is the soluble antigen identical with the RNA core of the virus, like in the influenza system, and if you purify this soluble antigen—let us suppose you centrifugate the virus and the soluble antigen is in the supernatant—and if you immunize against the soluble antigen will you get antibody? Did you ever try that? In the influenza system you can have antibody against this soluble antigen, which can be identified by complement fixation only. It will not neutralize the virus. It will recognize the type of the virus, but it will not distinguish between members within the type of influenza A viruses.

Dr. Aoki: We have considered the possibility that the soluble antigen is in fact the counterpart of the s-antigen of myxoviruses and we are doing experiments to elucidate this question. We now have precipitating G antisera that will be particularly useful for this purpose.

Dr. Lilly: Dr. Aoki, you said that in the serum of animals with leukemia induced by Gross virus you cannot find Friend, Moloney, or Rauscher (FMR) antigens. I don't think you said the contrary. Have you examined animals with leukemia induced by Friend, Rauscher, or Moloney for the G antigen?

Dr. Aoki: Yes. We find leukemias induced by Friend, Moloney, and Rauscher viruses can weakly absorb G antibody, whereas leukemias induced by Gross virus, including spontaneous leukemias of G+ strains, do not absorb FMR activity. This may be interpreted in two ways. First, preparations of Friend, Moloney, and Rauscher viruses may contain Gross virus, which in view of the exceedingly wide distribution of Gross virus is by no means unlikely. Second, antigens of the FMR group may share a determinant with antigens induced by Gross virus.

Dr. Bianco: Are soluble antigens detectable in the plasma of mice infected with leukemia virus, but which are not clearly leukemic yet?

Dr. Aoki: Yes. We find soluble antigen in all mice of G+ strains, and by this means we can distinguish mice of low-incidence strains from mice of high-incidence strains. In contrast, the serum of certain mice of G— strains contains natural G antibody. We have never found G antibody in mice of the high-incidence strains.

Further Studies on the Biological Relationship of Murine Leukemia Viruses and on Kidney Lesions of Mice With Leukemia Induced by These Viruses[1, 2]

Louis Recher,[3] Toshio Tanaka, John A. Sykes, Tokichi Yumoto,[4] Gabriel Seman,[5] Landrum Young, *and* Leon Dmochowski, *Department of Virology, University of Texas M. D. Anderson Hospital and Tumor Institute, and Department of Microbiology, Baylor University College of Medicine, Texas Medical Center, Houston, Texas*

SOME of the results of experiments designed to study the effect of inoculation of some murine leukemia viruses into mice of strains with a low and with a high incidence of spontaneous leukemia have already been reported (*1*). Glomerulonephritis detected by light microscopy in leukemic AKR mice inoculated with Friend or Rauscher virus had been an incidental finding in these earlier experiments. This glomerular lesion had been found exclusively in AKR mice with advanced erythroid leukemia and had not been observed in animals with early erythroid leukemia nor in mice failing to develop leukemia after inoculation of Friend or Rauscher virus. In these earlier experiments, glomerulonephritis was not observed in BALB/c mice with erythroid leukemia induced by Friend or Rauscher virus. This lesion was also not observed in BALB/c and AKR mice with lymphatic leukemia induced by Passage A (Gross) or Moloney virus.

In an attempt to explore the origin of glomerulonephritis observed in mice with virus-induced leukemia, particular attention was paid to this lesion in additional experiments on the biological relationship of murine leukemia viruses. The findings of these experiments are reported herein. The glomerulonephritis was again observed in some leukemic animals.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported in part by Public Health Service grant CA-04140-6 and by Public Health Service contract 43-65-585 from the National Cancer Institute, and by grant E-94-E from the American Cancer Society, Inc.

[3] Fellow of the Leukemia Society, Inc.

[4] Fellow of the China Medical Board of New York, Inc.

[5] Lilly International Fellow.

This provided an opportunity to examine the renal lesion in the electron microscope. The results of the electron microscope studies will be described.

MATERIALS AND METHODS

AKR/Dm/TEX[6] high-leukemia-strain and BALB/c/Dm/TEX[6] low-leukemia-strain mice, when 4 to 9 days old, were inoculated intraperitoneally with 0.2 ml of a cell-free, undiluted 20% w/v leukemic tissue homogenate virus preparation of Passage A (Gross), Moloney, Friend, or Rauscher virus or of a preparation of these viruses in dilutions varying from 10^{-1} to 10^{-5}. In addition, 4- to 9-day-old BALB/c mice were inoculated 24 hours apart with 0.2 ml of the following virus preparations in a 10^{-2} dilution: Passage A and Friend; Passage A and Rauscher; Moloney and Friend; Moloney and Rauscher; Passage A and Moloney; and Friend and Rauscher.

Virus strain.—Passage A (Gross) virus, originally received from Dr. L. Gross, was passaged in C3Hf/Bi/Gr/Dm mice in our laboratory. The term "Passage A (Original) virus" has been introduced to identify Gross virus obtained from organs of C3Hf/Bi/Gr/Dm mice with leukemia. The term "Passage A (AKR) virus" has been used to identify virus preparations obtained from AKR/Dm/TEX mice with leukemia. The term "Passage A (AKR) virus" has been used to identify virus preparations obtained from AKR/Dm/TEX mice with leukemia induced by Passage A (Original) virus.

Moloney virus, received from Dr. J. B. Moloney, was passaged in BALB/c/Dm/TEX mice. The term "Moloney (Original) virus" has been used to designate virus preparations from organs of BALB/c/Dm/TEX mice with leukemia. The term "Moloney (AKR) virus" has been introduced to designate virus preparations obtained from organs of AKR/Dm/TEX mice with leukemia induced by Moloney (Original) virus.

Friend virus, obtained from Dr. Charlotte Friend, was passaged in BALB/c/Dm/TEX mice. The term "Friend (Original) virus" denotes virus preparations derived from leukemic organs of BALB/c/Dm/TEX mice. "Friend (AKR) virus" denotes virus preparations derived from organs of AKR/Dm/TEX mice with leukemia induced by Friend (Original) virus

Rauscher virus, obtained from Dr. F. J. Rauscher, was passaged in BALB/c/Dm/TEX mice. The term "Rauscher (Original) virus" designates virus preparations obtained from organs of leukemic BALB/c/Dm/TEX mice. The term "Rauscher (AKR)" designates virus preparations derived from organs of AKR/Dm/TEX mice with leukemia induced by Rauscher (Original) virus.

---

[6] Obtained from Texas Inbred Mice Co., 305 Almeda-Genoa Road, Houston, Texas.

The virus-inoculated mice were kept for 18 months or until they showed signs of leukemia. Tissues taken for light microscope study included spleen, liver, thymus, lymph nodes, lung, pancreas, salivary gland, uterus, ovary, and testicles. The tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections from all tissue samples were stained with hematoxylin and eosin. Selected sections of the kidneys were also stained with periodic acid-Schiff, Congo red, and phosphotungstic acid-hematoxylin stains.

Kidney tissue was removed and processed for electron microscopy from the following mice: 4 AKR/Dm/TEX mice and 1 BALB/c/Dm/TEX mouse with erythroid leukemia induced by Friend (Original) virus; 2 BALB/c/Dm/TEX mice with erythroid leukemia induced by Rauscher (Original) virus; 1 BALB/c/Dm/TEX mouse with lymphatic leukemia induced by Moloney (Original) virus; and 1 BALB/c/Dm/TEX mouse with lymphatic leukemia induced by Passage A (Original) virus. As a control, kidney tissue from 2 AKR/Dm/TEX mice with spontaneous lymphatic leukemia and from 2 nonleukemic BALB/c/Dm/TEX mice and 2 nonleukemic AKR/Dm/TEX mice (10 weeks old) was taken.

Tissues for electron microscopic studies were cut into small pieces and fixed in the cold with 2% osmic acid buffered with sodium and potassium phosphate (*p*H 7.2) with added glucose (*2*). The tissue was dehydrated in a series of graded ethyl alcohols (for 30 minutes in each) varying from 30 to 100%, treated with a mixture of absolute ethyl alcohol and *n*-butylmethacrylate (equal volumes) for 1 hour, and then placed in *n*-butylmethacrylate for 1 hour. Thereafter the tissue was treated with *n*-butylmethacrylate with 1% benzoyl peroxide for 1 hour and embedded in prepolymerized *n*-butylmethacrylate with 1% benzoyl peroxide at 56° C for 24 hours. In addition, some tissues were fixed in 3% glutaraldehyde for 2 hours, washed in phosphate buffer overnight, post-fixed in 2% buffered (sodium and potassium phosphate) osmic acid for 2 hours, washed, dehydrated in graded alcohols, and embedded in *n*-butylmethacrylate. Sections were cut with glass knives on a Porter-Blum (Sorvall) ultramicrotome, stained with a saturated solution of uranyl acetate for 2 hours, washed in triple distilled water, and counterstained with a solution of lead tartrate for 10 minutes (*3*). Sections were then coated with a thin film of carbon and examined with an RCA EMU 3G electron microscope at 80 or 100 kv. Original magnifications of the electron micrographs varied from 7,000 to 15,000 diameters.

RESULTS

In the experiments on the relationship of murine leukemia viruses, all leukemias observed were either of the lymphatic or of the erythroid type.

The results of experiments carried out with Passage A (Original) and Passage A (AKR) virus preparations are summarized in table 1. Passage

Table 1.—Results of inoculation of AKR and BALB/c mice with Passage A (Gross) virus preparation

Source of virus	Strain of mice inoculated	Dilution	Number of mice alive at the earliest time of leukemia appearance	Number of mice developing leukemia	Average time of appearance of leukemia (days)	Duration of experiment (days)
C3H/f (Original)	BALB/c	10^0	27*	—	—	242† and 290†
		10^{-1}	34	2 (6%)	209	235† and 290†
		10^{-2}	20*	—	—	328†
		10^{-3}	26*	—	—	328†
		10^{-4}	21*	—	—	334†
		10^{-5}	19*	—	—	334†
	AKR	10^{-1}	28	21 L‡ (95%)	255	Completed
		10^{-2}	25	21 L (84%)	222	Completed
		10^{-3}	20*	—	—	154†
		10^{-4}	13	4 L (31%)	271	Completed
		10^{-5}	11	1 L (9%)	140	168†
AKR (AKR)	BALB/c	10^0	9	1 L (11%)	153	161†
		10^{-1}	29*	—	—	156†
		10^{-2}	27*	—	—	161†
		10^{-3}	14	1 L (7%)	397	Completed
		10^{-4}	16*		—	159†
		10^{-5}	18*		—	158†
	AKR	10^0	22	3 L (14%)	80	174†
		10^{-1}	24	2 L (8%)	125	183†
		10^{-2}	14	6 L (43%)	268	Completed
		10^{-3}	13	8 L (61%)	272	Completed
		10^{-4}	7	7 L (100%)	265	Completed
		10^{-5}	8	6 L (75%)	283	Completed

*Number of mice weaned.
†Experiments still in progress.
‡L denotes lymphatic leukemia.

A (Original) and Passage A (AKR) virus induced lymphatic leukemia in only a few BALB/c mice. A higher incidence of lymphatic leukemia was observed in AKR mice inoculated with the same virus (Original or AKR), but the average time of appearance of leukemia calculated for all experiments was 240 days.

The results of experiments carried out with Moloney (Original) and Moloney (AKR) virus preparations are summarized in table 2. In BALB/c mice inoculated with Moloney (Original) virus, lymphatic leukemia occurred in 50 to 90% of the animals within an average time of 120 days. BALB/c mice inoculated with Moloney (AKR) virus developed lymphatic leukemia only when the virus preparation was undiluted or diluted 10^{-1}. The virus preparation, undiluted and diluted 10^{-1}, induced leukemia in 50% and 15% of the BALB/c mice, respectively, with an average time of appearance of leukemia of 135 and 166 days. So far, virus preparations in higher dilutions have failed to induce leukemia. Moloney (Original) virus inoculated in different dilutions into AKR mice induced lymphatic leukemia in 54 to 83% of the animals. In all but one experiment where the average latency time was 191 days, the average time of appearance was 300 days. Moloney (AKR) virus inoculated into AKR mice induced lymphatic leukemia in up to 83% of the animals; the average time of appearance of leukemia was 300 days.

The results of experiments carried out with Friend (Original) and Friend (AKR) virus preparations are summarized in table 3. Friend (Original) virus inoculated in dilutions varying from 10^{0} to 10^{-3} into BALB/c mice induced erythroid leukemia in more than 65% of the animals. The average time of appearance of leukemia ranged from 50 to 90 days. The incidence of leukemia was lower when Friend (Original) virus preparation in higher dilutions was used. Friend (AKR) virus inoculated into BALB/c mice induced a lower incidence of erythroid leukemia compared with that observed in BALB/c mice inoculated with Friend (Original) virus. While the incidence of erythroid leukemia was 100% in BALB/c mice inoculated with an undiluted Friend (AKR) virus preparation, the incidence of leukemia was remarkably lower in mice of the same strain inoculated with higher dilutions of Friend (AKR) virus preparation. Friend (Original) virus inoculated into AKR mice induced erythroid leukemia in 80 to 100% of the animals when the virus preparation was diluted 10^{-1} to 10^{-3}. The incidence of erythroid leukemia in AKR mice was significantly lower when higher dilutions of Friend (Original) virus preparation were used. Interestingly, up to 55% of the AKR mice developed lymphatic leukemia after an average latency period of 186 to 238 days. Only 13% of the AKR mice inoculated with Friend (AKR) virus preparation developed erythroid leukemia, but up to 45% of the AKR mice developed lymphatic leukemia after an average latency period of 280 days.

The results of experiments carried out with Rauscher (Original) and Rauscher (AKR) virus preparations are summarized in table 4. Rauscher

TABLE 2.—Results of inoculation of AKR and BALB/c mice with Moloney virus preparation

Source of virus	Strain of mice inoculated	Dilution	Number of mice alive at the earliest time of leukemia appearance	Number of mice developing leukemia	Average time of appearance of leukemia (days)	Duration of experiment (days)
BALB/c (Original)	BALB/c	10^0	33	24 L* (73%)	121	Completed
		10^{-1}	22	11 L (50%)	111	Completed
		10^{-2}	13	11 L (85%)	113	Completed
		10^{-3}	20	18 L (90%)	135	Completed
		10^{-4}	11	8 L (73%)	130	Completed
		10^{-5}	9	5 L (55%)	134	Completed
	AKR	10^{-1}	16	9 L (56%)	287	Completed
		10^{-2}	15	10 L (66%)	289	Completed
		10^{-3}	17	13 L (80%)	191	Completed
		10^{-4}	18†	—	—	189‡
		10^{-5}	23	4 L (18%)	158	202‡
AKR (AKR)	BALB/c	10^0	18	9 L (50%)	135	340‡
		10^{-1}	13	2 L (15%)	166	346‡
		10^{-2}	27†	—	—	203‡
		10^{-3}	24†	—	—	208‡
		10^{-4}	20†	—	—	215‡
		10^{-5}	17†	—	—	215‡
	AKR	10^0	28	7 L (25%)	145	211‡
		10^{-1}	26†	—	—	215‡
		10^{-2}	6	5 L (83%)	298	Completed
		10^{-3}	24	13 L (54%)	393	Completed
		10^{-4}	20	14 L (70%)	305	Completed

*L denotes lymphatic leukemia.
†Number of mice weaned.
‡Experiments still in progress.

TABLE 3.—Results of inoculation of AKR and BALB/c mice with Friend virus preparation

Source of virus	Strain of mice inoculated	Dilution	Number of mice alive at the earliest time of leukemia appearance	Number of mice developing leukemia	Average time of appearance of leukemia (days)	Duration of experiment (days)
BALB/c (Original)	BALB/c	10^0	11	10 E*(91%)	50	Completed
		10^{-1}	12	8 E (66%)	90	Completed
		10^{-2}	6	6 E (100%)	50	Completed
		10^{-3}	31	23 E (74%)	82	304†
		10^{-4}	39	20 E (52%)	87	235† and 255†
		10^{-5}	19	4 E (21%)	120	314†
	AKR	10^{-1}	5	4 E (80%)	47	Completed
		10^{-2}	16	16 E (100%)	34	Completed
		10^{-3}	31	25 E (81%)	35	Completed
		10^{-4}	38	9 E (24%)	41	237†
				7 L‡(19%)	238	
		10^{-5}	11	5 E (45%)	73	243†
				6 L (55%)	186	
AKR (AKR)	BALB/c	10^0	14	14 E (100%)	59	Completed
		10^{-1}	9	2 E (22%)	144	Completed
		10^{-2}	17	3 E (18%)	70	111†
		10^{-3}	33	5 E (15%)	66	110† and 147†
		10^{-4}	17§	—	—	147†
		10^{-5}	42§	—	—	Completed
	AKR	10^{-2}	22	10 L (45%)	280	Completed
		10^{-3}	53	18 L (34%)	286	Completed
				7 E (13%)	90	
		10^{-4}	28	11 L (13%)	281	Completed
				1 E (3.5%)	64	

*E denotes erythroid leukemia.
†Experiments still in progress.
‡L denotes lymphatic leukemia.
§Number of mice weaned.

Table 4.—Results of inoculation of AKR and BALB/c mice with Rauscher virus preparation

Source of virus	Strain of mice inoculated	Dilution	Number of mice alive at the earliest time of leukemia appearance	Number of mice developing leukemia	Average time of appearance of leukemia (days)	Duration of experiment (days)
BALB/c (Original)	BALB/c	10^{0}	13	9 E* (70%)	35	112†
		10^{-1}	50	36 E (72%)	68	112† and 154†
		10^{-2}	35	12 E (34%)	58	103†
		10^{-3}	27	9 E (33%)	135	152†
		10^{-4}	12	3 E (25%)	256	280†
		10^{-5}	18‡	—	—	280†
	AKR	10^{0}	10	10 E (100%)	49	Completed
		10^{-1}	16	10 E (62%)	52	Completed
		10^{-2}	26	17 L§ (65%)	250	Completed
		10^{-3}	25	11 L (44%)	308	Completed
AKR (AKR)	BALB/c	10^{0}	23	1 E (4%)	41	143†
		10^{-1}	18	13 E (72%)	58	299†
		10^{-2}	11	6 E (55%)	154	299†
		10^{-3}	5	1 L (20%)	237	Completed
		10^{-4}	12	4 E (33%) 1 L (8%)	195 384	Completed
		10^{-5}	25‡	—	—	224†
	AKR	10^{-1}	10	6 E (60%)	189	404†
		10^{-2}	8	3 L (38%)	293	404†
		10^{-3}	28	13 L (46%) 1 E (3.5%)	260 62	Completed
		10^{-4}	19	5 L (26%) 1 E (5%)	282 177	Completed

*E denotes erythroid leukemia.
†Experiments still in progress.
‡Number of mice weaned.
§L denotes lymphatic leukemia.

(Original) virus inoculated into BALB/c mice induced erythroid leukemia in up to 72% of the animals within an average time of 68 days. Rauscher (AKR) virus inoculated into BALB/c mice induced erythroid leukemia in up to 72% of the animals within an average time of 58 days, but 2 animals inoculated with Rauscher (AKR) virus in a 10^{-3} and 10^{-4} dilution developed lymphatic leukemia. Rauscher (Original) virus inoculated into AKR mice induced a high incidence (up to 100%) of erythroid leukemia when the virus preparation was used in 10^{0} and 10^{-1} dilutions. Up to 65% of the AKR mice inoculated with Rauscher (Original) virus in 10^{-2} and 10^{-3} dilutions developed lymphatic leukemia at an average time of 250 to 308 days. Rauscher (AKR) virus inoculated into AKR mice induced in some mice erythroid leukemia and in others lymphatic leukemia. Of AKR mice inoculated with Rauscher (AKR) virus in a 10^{-1} dilution, 60% developed erythroid leukemia after an average latency time of 189 days. In most AKR mice, the virus preparations in higher dilutions induced lymphatic leukemia at an average latency time of 270 days.

In table 5 the results of experiments on interference are shown. As may be seen, 40% of the BALB/c mice inoculated with Moloney virus preparation followed 24 hours later by an inoculation of Passage A virus developed lymphatic leukemia after an average latency time of 102 days. Inoculation of Friend virus preparation followed by Rauscher virus preparation induced in 100% of the BALB/c mice erythroid leukemia, with an average time of 35 days. Inoculation of Passage A virus preparation followed by Friend or Rauscher virus preparation induced erythroid

TABLE 5.—Results of inoculation of BALB/c mice with 2 murine leukemia viruses 24 hours apart

Strain of virus inoculated	Dilution	Number of mice alive at the earliest time of leukemia appearance	Number of mice developing leukemia	Average time of appearance of leukemia (days)
Moloney (Original)— Passage A (Original)	10^{-2}	47	19 L*(40%)	102
Friend (Original)— Rauscher (Original)	10^{-2}	15	15 E†(100%)	35
Passage A (Original)— Friend (Original)	10^{-2}	27	10 E (37%)	44
Moloney (Original)— Friend (Original)	10^{-2}	28	22 E (78%) 1 L (3%)	46 76
Passage A (Original)— Rauscher (Original)	10^{-2}	34	20 E (60%) 1 L (3%)	59 59
Moloney (Original)— Rauscher (Original)	10^{-2}	12	1 E (8%) 6 L (50%)	42 120

*L denotes lymphatic leukemia.
†E denotes erythroid leukemia.

leukemia in all BALB/c mice that developed leukemia, except one which developed lymphatic leukemia after Rauscher virus inoculation. Moloney virus followed by inoculation of Friend virus preparation induced erythroid leukemia in all animals with leukemia except one which developed lymphatic leukemia. Inoculation of Moloney virus followed by Rauscher virus preparation induced lymphatic leukemia in all BALB/c mice showing leukemia except one which developed erythroid leukemia.

STUDIES ON GLOMERULONEPHRITIS

In previous studies (*1*), the description of renal glomerular lesions had been based entirely on light microscopic examination. In the present experiments, correlative electron microscope studies have shown that renal glomerular lesions not only occur in AKR mice with erythroid leukemia, induced by Friend or Rauscher virus, but also in BALB/c mice with erythroid leukemia induced by either Friend or Rauscher virus preparations. Glomerular changes have also been found in AKR mice with spontaneous lymphatic leukemia. The findings obtained by electron microscopy led to close examination of the kidneys by the light microscope for minor glomerular changes. This examination has revealed glomerular changes in AKR mice with spontaneous leukemia, in BALB/c mice with erythroid leukemia induced by Friend or Rauscher virus, and in BALB/c mice with lymphatic leukemia induced by Passage A virus. Although glomerular changes have been observed in BALB/c mice with induced leukemia and in AKR mice with spontaneous leukemia, they have never been as frequent and as severe as the lesions observed in AKR mice with erythroid leukemia induced by Friend or Rauscher virus.

The earliest changes seen in sections stained by hematoxylin and eosin were characterized by hyaline thickening of the walls of glomerular capillaries with narrowing of the capillary lumina. Fibrinoid thrombi were occasionally seen in capillary lumina. They appeared dark blue, indicating presence of fibrin, in sections stained with phosphotungstic acid and hematoxylin. In more advanced glomerular lesions, capillary loops appeared to have melted together, converting the glomerulus into a lobulated hyaline and fibrinoid mass with total obliteration of the glomerular fine structure. In no instance has any crescent formation of the Bowman capsule been observed nor any adhesions between Bowman capsule and glomerulus. The hyaline and fibrinoid material of obliterated glomeruli appeared purple (PAS-positive) in sections stained with periodic acid-Schiff. Sections stained with Congo red showed no evidence of amyloid.

Glomerular lesions observed in AKR mice with leukemia induced by Friend virus are illustrated in figures 1, 2, and 3. Figure 4 shows a glomerular lesion of a BALB/c mouse with erythroid leukemia induced by Rauscher virus. Figure 5 shows a glomerular lesion of a BALB/c mouse with leukemia induced by Passage A virus, and figure 6 shows a glomerular lesion in an AKR mouse with spontaneous lymphatic leukemia.

Most of the electron microscope studies on renal lesions have been carried out on 4 AKR mice with erythroid leukemia induced by Friend (Original) virus. In this group, 2 AKR mice showed severe changes and 2 only minor changes in the light microscope. All 4 mice showed glomerular changes in the electron microscope. The glomerular changes were characterized by an obliterative process of the glomerular capillaries with the presence, often in large numbers, of mature leukemia virus particles within capillaries. The leukemia virus particles were embedded in proteinaceous material of varying electron density or in a homogenous, finely granular substance resembling lipid. Endothelial cells of glomerular capillaries had varying degrees of degeneration. The basement membranes often appeared to have merged with proteinaceous material deposited on their inner surface, with loss of their distinct outline. While the entire process primarily appeared to be an intracapillary process leading to obliteration of the capillary lumen, in severe lesions degenerative changes were also present in epithelial cells (podocytes). Similar glomerular changes have also been found in BALB/c mice with erythroid leukemia induced with Friend or Rauscher virus and in AKR mice with spontaneous lymphatic leukemia. However, smaller numbers of leukemia virus particles were found in mice of these strains. BALB/c mice with lymphatic leukemia induced by Moloney or Passage A virus have so far failed to show this lesion in the electron microscope, although it has been found by light microscopy in a BALB/c mouse with lymphatic leukemia induced by Passage A virus.

In no instance have mature leukemia virus particles been found outside the lumen of glomerular capillaries nor have any immature or budding virus particles been observed.

Mature virus particles within a substance resembling lipid are shown in figures 7 and 8. The virus particles are located between the basement membrane and the nuclear zone of an endothelial cell. A partially obliterated glomerular capillary including small groups of leukemia virus particles is shown in figure 9. Groups of leukemia virus particles within dense proteinaceous material may be seen in figures 10 and 11 in which the capillary lumen appears obliterated. In figure 11 the basement membrane shows loss of its usual distinct outline. Degenerative changes of the adjacent epithelial cell are also present. In 2 control 10-week-old nonleukemic AKR mice and in 2 BALB/c mice, glomerular lesions could not be found in the light microscope or in the electron microscope.

DISCUSSION

Gross (*4*) has shown that BALB/c mice are susceptible to Passage A virus. He found an incidence of 88% of leukemia in his BALB/c mice inoculated with Passage A virus. In the present experiments BALB/c mice showed an extremely poor response to the inoculation of Passage A (Original) and Passage A (AKR) virus. A higher incidence of leukemia occurred in AKR mice, but with an average latent period of 240 days. Of the control AKR mice, 67% developed spontaneous leukemia with an average latent period of 300 days. Inoculation of Passage A (Original) and Passage A (AKR), therefore, did not significantly reduce the latent period of spontaneous leukemia in AKR mice. The poor response of BALB/c mice to inoculation of Passage A virus and the failure of the same virus to reduce the latent period of spontaneous leukemia in AKR mice by a factor of 2 to 3, as reported by Rudali *et al.* (*5*), indicate that the Passage A virus used in these experiments had a low-leukemia-inducing activity.

Moloney and Friend virus after a single passage in AKR mice showed a remarkable loss of leukemia-inducing activity when inoculated into BALB/c mice. When Friend or Rauscher virus after a single passage in AKR mice was reinoculated into mice of the same strain, the leukemia-inducing activity was significantly reduced. While AKR mice appeared to be susceptible to all 3 viruses—Moloney, Friend, and Rauscher—a loss of leukemia-inducing activity was noted when these 3 viruses after a single passage were reinoculated into BALB/c or AKR mice. The question therefore arises whether these viruses could be maintained in AKR mice by serial passage. Experiments are now in progress in an attempt to demonstrate the type of leukemia-inducing activity of Friend and Rauscher virus following repeated passages in AKR mice.

Studies on possible interference between the 4 murine leukemia viruses examined have shown some unexpected results: A low incidence of lymphatic leukemia was found in BALB/c mice inoculated with Moloney virus followed 24 hours later by inoculation of Passage A virus (tables 2 and 5). There was a low incidence of erythroid leukemia when inoculation of Passage A virus was followed by inoculation of Friend virus (table 5). The possibility that interference might have occurred in these two experiments must be considered. BALB/c mice had a high incidence of erythroid leukemia when inoculation of Friend virus followed that of Moloney virus. Inoculation of Rauscher virus after the inoculation of Moloney virus resulted in a high incidence of lymphatic leukemia. These results are difficult to interpret without knowledge of the quantitative relationship of the 2 virus preparations inoculated (24 hours apart) into the same BALB/c mice, although the dilutions of virus preparations were comparable on gram-equivalent basis.

Glomerulonephritis in inbred C57BL mice more than 18 months old was described by Gorer (*6*). Kirschbaum *et al.* (*7*) reported a high incidence of spontaneous glomerulonephritis in NH (Minnesota subline) strain mice. Only female mice of this strain developed the renal lesion after a latency period of 11 to 20 months. It is evident that spontaneous glomerulonephritis in C57BL and inbred NH mice, as reported in these two communications, appears to have developed only in older mice (11 months and over) and apparently was not associated with leukemia. In the present study, glomerulonephritis has been observed in AKR and BALB/c mice with induced leukemia as early as 30 days after inoculation of Friend or Rauscher virus.

The frequent presence of large numbers of leukemia virus particles in the renal glomerular lesions brings up the question whether the leukemia virus is responsible for the development of this lesion. Such a view could be supported by the fact that usually more virus particles have been found in severe lesions than in glomeruli with mild changes. It appears that glomerulonephritis is associated with the development of manifestations of leukemia. Therefore, the leukemic process may at least in part be responsible for this lesion. Glomerulonephritis may be the result of an autoimmune reaction to a tissue antigen altered by viral infection.

SUMMARY

Passage A (Gross), Moloney, Friend, and Rauscher murine leukemia viruses were inoculated into BALB/c/Dm/TEX mice, known to have a very low incidence of spontaneous leukemia (0.1%), and into AKR/Dm/TEX mice, with a high incidence of spontaneous leukemia (83%). Moloney and Passage A viruses induced only lymphatic leukemia in BALB/c and AKR mice. Friend and Rauscher viruses induced leukemia of the erythroid cell system in mice of both strains. AKR mice were highly susceptible to induction of leukemia by Friend and Rauscher viruses.

The Passage A virus had a low-leukemia-inducing activity when inoculated into BALB/c/Dm/TEX and AKR/Dm/TEX mice.

Moloney, Friend, and Rauscher viruses showed, after a single passage in AKR mice, a loss of leukemia-inducing activity when inoculated into BALB/c/Dm/TEX mice or reinoculated into AKR/Dm/TEX mice.

By light microscopy, a glomerulonephritis was observed in AKR/Dm/TEX mice with erythroid leukemia induced by Friend or Rauscher virus and in AKR/Dm/TEX mice with spontaneous lymphatic leukemia. Glomerulonephritis was also observed in BALB/c/Dm/TEX mice with erythroid leukemia induced by Friend or Rauscher virus and in BALB/c/Dm/TEX mice with lymphatic leukemia induced by Passage A (Gross) virus. Correlative electron microscope studies have shown leukemia virus particles (mature type) in such renal glomerular lesions.

REFERENCES

(*1*) Dmochowski, L., Recher, L., Tanaka, T., Yumoto, T., Sykes, J. A., and Young, L.: Studies on the biological relationship of some murine leukemia viruses. Cancer Res 26: 382–394, 1966.

(2) Millonig, G.: Advantages of a phosphate buffer for OsO_4 solution in fixation. Proc 19th Ann Meeting Electron Microscope Soc Amer, 1961, pp 15–16.

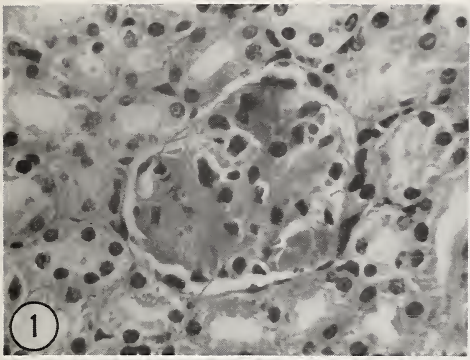
(3) ———: A modified procedure for lead staining of thin sections. J Biophys Biochem Cytol 11: 736–739, 1961.

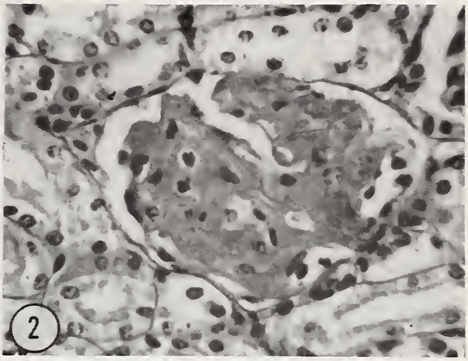
(4) Gross, L.: Pathogenic potency and host range of the mouse leukemia virus. Acta Haemat (Basel) 29: 1–15, 1963.

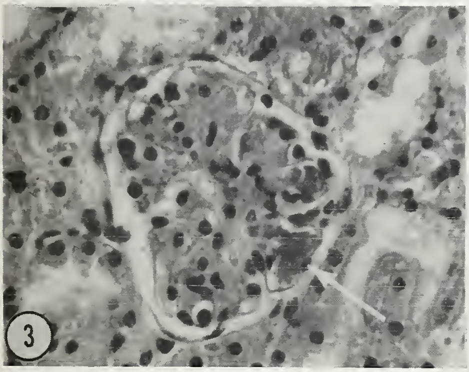
(5) Rudali, G., Duplan, J. F., and Latarjet, R.: Leucémogénèse chez des souris AK injectées à la naissance avec un extrait leucémique aqueux isologue. Bull Cancer 44: 440–443, 1957.

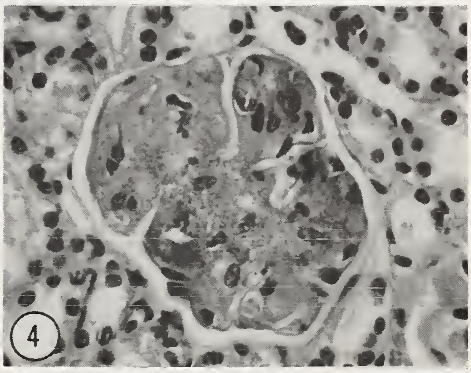
(6) Gorer, P. A.: Renal lesions found in pure lines of mice. J Path Bact 50: 25–30, 1940.

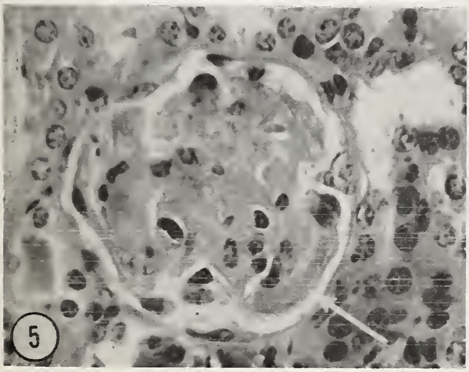
(7) Kirschbaum, A., Bell, E. T., and Gordon, J.: Spontaneous and induced glomerulonephritis in an inbred strain of mice. J Lab Clin Med 34: 209–220, 1949.











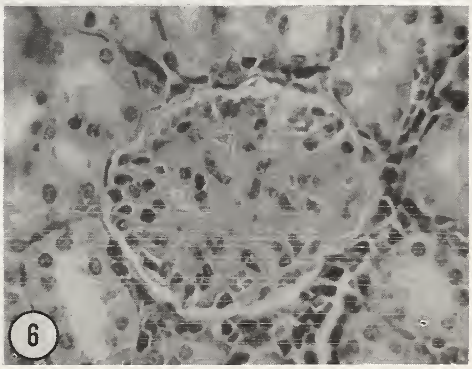


FIGURE 1.—Kidney from AKR mouse with erythroid leukemia induced by Friend virus, showing hyalinization of glomerulus with obliteration of capillaries. Hematoxylin and eosin. × 340

FIGURE 2.—Kidney from same mouse as in figure 1. Periodic acid-Schiff (strongly positive). × 340

FIGURE 3.—Kidney from AKR mouse with erythroid leukemia induced by Friend virus, showing moderate hyalinization of glomerulus with fibrinoid thrombus in capillary loop (*arrow*). Hematoxylin and eosin. × 340

FIGURE 4.—Kidney from BALB/c mouse with erythroid leukemia induced by Rauscher virus, showing lobulated obliterated glomerulus. Hematoxylin and eosin. × 340

FIGURE 5.—Kidney from BALB/c mouse with lymphatic leukemia induced by Passage A (Gross) virus, showing obliterated glomerulus with fibrinoid thrombus in one capillary loop (*arrow*). Hematoxylin and eosin. × 340

FIGURE 6.—Kidney from AKR mouse with spontaneous lymphatic leukemia, showing obliterated glomerulus. Hematoxylin and eosin. × 340

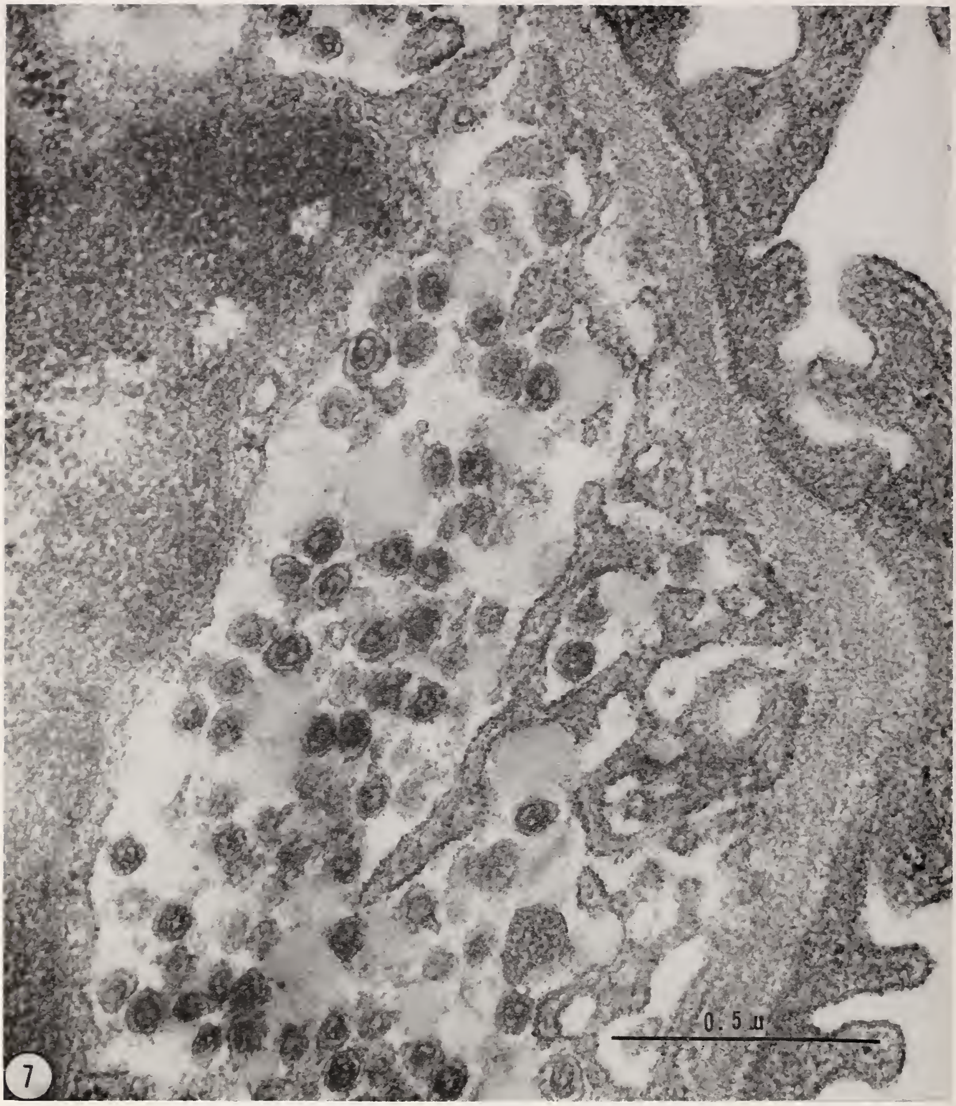


FIGURE 7.—Section of renal glomerulus from AKR mouse with erythroid leukemia induced by Friend (Original) virus. Mature leukemia virus particles may be seen within pale amorphous substance (lipid ?) between basement membrane and nuclear zone of endothelial cell. × 75,000

8

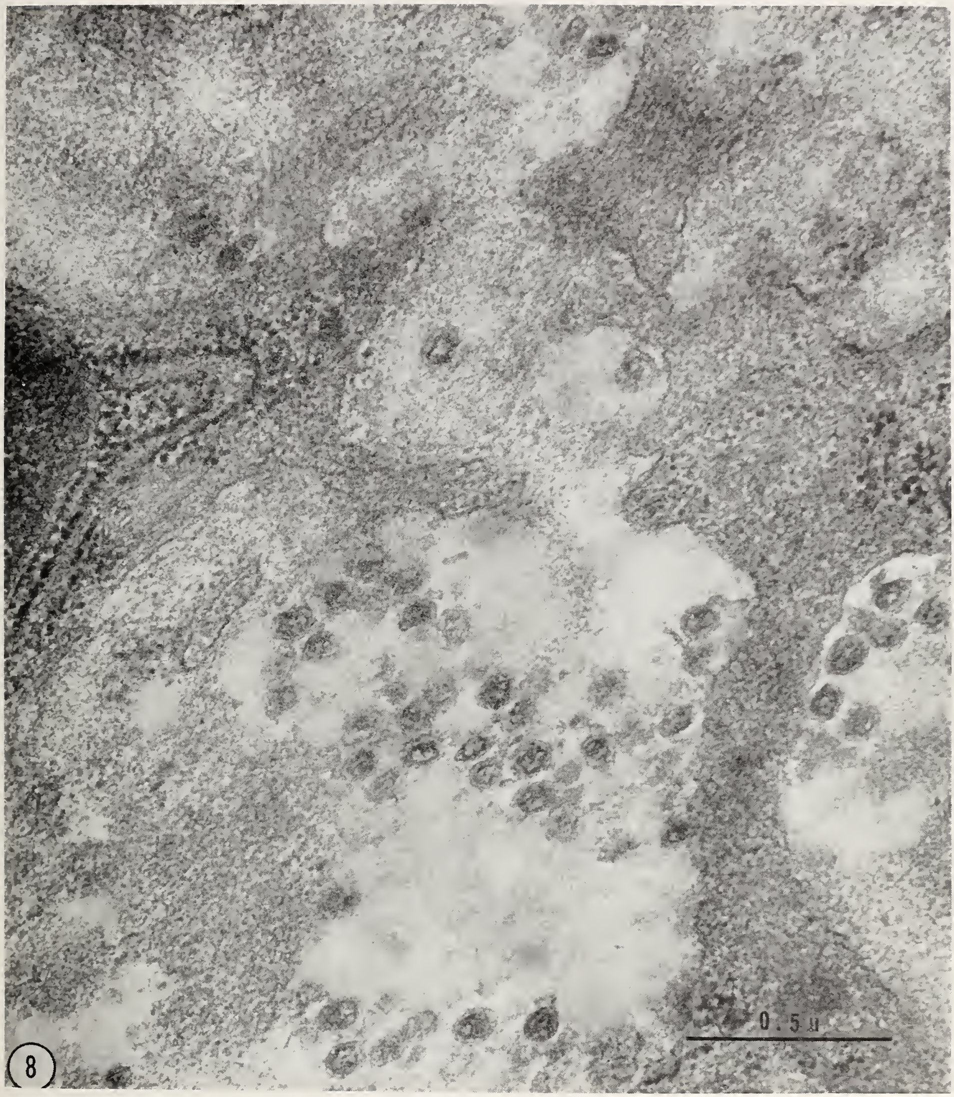


FIGURE 8.—Section of renal glomerulus from AKR mouse with erythroid leukemia induced by Friend (Original) virus. Mature leukemia virus particles within pale amorphous substance and dense granular material in an obliterated capillary. × 60,000

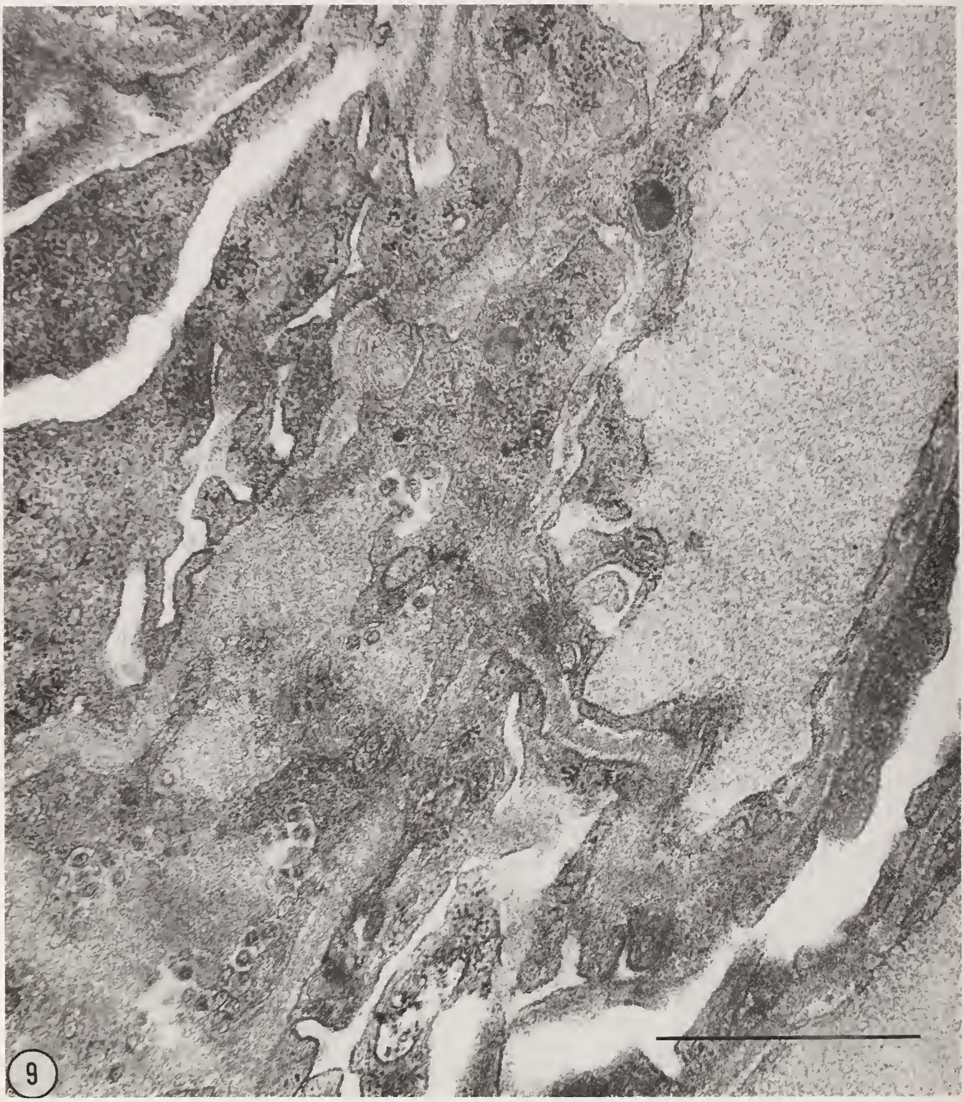


FIGURE 9.—Section of renal glomerulus from AKR mouse with erythroid leukemia induced by Friend (Original) virus. A partially obliterated capillary including small groups of mature leukemia virus particles is shown. × 35,000

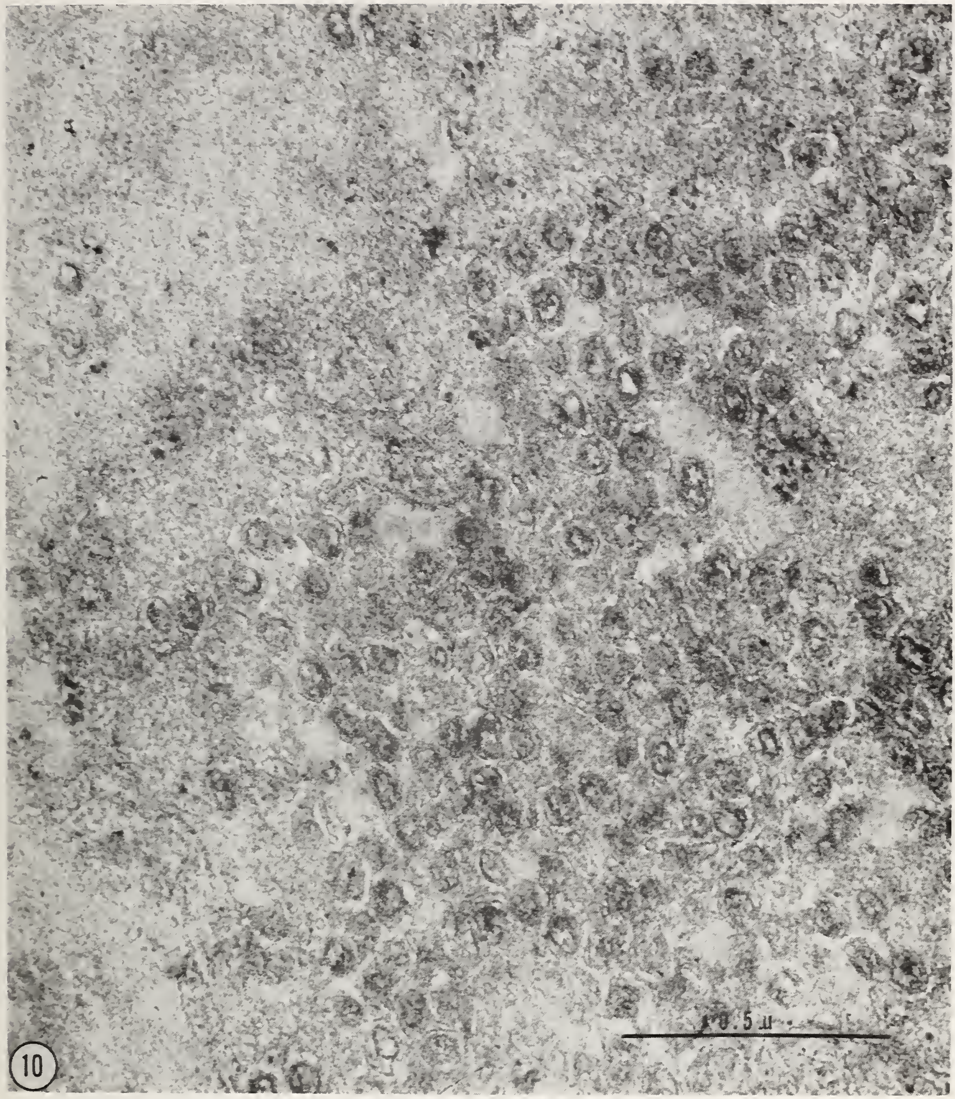


Figure 10.—Section of renal glomerulus from AKR mouse with erythroid leukemia induced by Friend (Original) virus. Large numbers of mature leukemia virus particles present within dense granular material. × 75,000

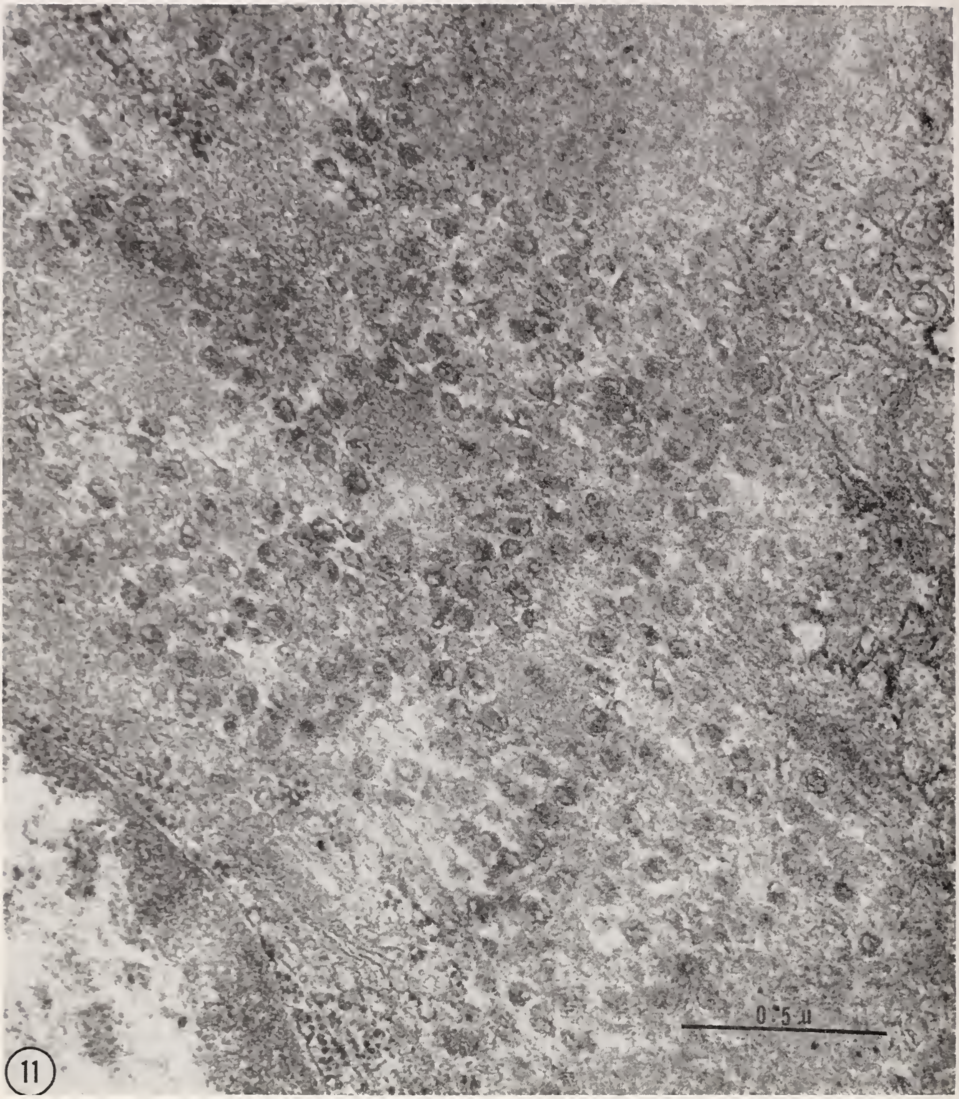


FIGURE 11.—Section of renal glomerulus from AKR mouse with erythroid leukemia induced by Friend (Original) virus. Large numbers of mature leukemia virus particles may be seen. Degeneration of basement membrane present. × 60,000

DISCUSSION

Dr. Dunn: I am very much interested in your discussion. We certainly see the same reactions in our Rauscher virus-infected mice, but we do not quite interpret them the same way. We are still very reluctant to call this an erythroleukemia. Let us excuse the uncertainty among pathologists by admitting that this is something new in our experience and we have to adjust to it, but we have felt that this virus reaction still belongs to some class of proliferative diseases.

I have been particularly interested, though, in the report on the glomerular lesion. Just before this meeting I was talking with Dr. Mirand and he has seen a similar lesion in some of his mice treated with the Friend virus. We have seen it particularly in Rauscher-infected mice kept alive for a long time by putting propylthiouracil in the food, thereby delaying the proliferative process, and most of these mice showed this type of glomerular lesion. We have not had an opportunity to see the glomeruli by electron microscopy, and I am delighted to have seen these pictures here.

Dr. Rappaport: I was pleased that Dr. Recher used both tissue section and imprint method for accurate classification of this lesion. This is often necessary and by using both methods he was able to dispel the concept that Friend's disease might be a reticulum cell sarcoma. It is indeed a purely erythroblastic proliferation.

I would like, however, to ask what the bone marrow showed in these mice, since Dr. Recher placed such emphasis on this being a systemic hematopoietic disease.

Dr. Recher: In all advanced cases of leukemia I could find groups of large "reticulum cells" normally not seen in sections of bone marrow of nonleukemic mice.

Friend and Rauscher diseases have impressed me as being well differentiated and not too aggressive neoplasms of the erythropoietic system, showing in the early stage proliferation of cells indistinguishable from normal erythroblasts. The normal bone marrow of mice is crowded with hematopoietic cells including erythroblasts and, unlike human bone marrow, contains no fat tissue which could possibly be replaced by leukemic infiltrates. For these reasons, leukemic infiltration of the bone marrow might not occur in the early stage, or if it does occur, it would be difficult to recognize. In the advanced stage, however, when immature cells ("reticulum cells") are predominant in leukemic infiltrates—to be expected since these cells most likely have a higher mitotic rate—infiltration of the bone marrow is more likely to occur and can be recognized without difficulty.

Dr. Metcalf: Just to defend this story about reticulum cell sarcoma, since I think we are responsible for it, if you reread the original paper you will find it was only late in the disease and only in a certain percentage of survivors that reticulum cell sarcoma developed (Metcalf, D., Furth, J., and Buffett, R. F., Cancer Res. 19: 52, 1959). We strongly believe morphology is not enough to diagnose these tumors but should be combined with functional tests. In this specific case, the large cells, in what we described as reticulum cell sarcoma, were found to be phagocytic, which is not a property of a primitive erythroid cell.

Antigenic Behavior of Moloney Virus-Induced Lymphomas in Mice[1]

GEORGE KLEIN *and* EVA KLEIN, *Department of Tumor Biology, Karolinska Institutet Medical School, Stockholm, Sweden*

A number of mouse lymphomas induced by the Moloney agent were compared with regard to: their sensitivity to the rejection response of syngeneic mice preimmunized with Moloney virus-containing materials ("immunosensitivity *in vivo*"); their susceptibility to the cytotoxic action of sera from resistant syngeneic animals ("immunosensitivity *in vitro*"); their ability to release virus, estimated by the mouse antibody production (MAP) test; and their concentration of tumor-specific cell-surface antigen, as judged by the indirect fluorescent antibody test and by absorption and inhibition experiments. The different parameters studied varied between different lymphomas, while individual lymphomas showed characteristic and well-reproducible patterns of behavior. All lymphomas examined carried the specific transplantation antigen characteristic for this system, although in different quantities. With one notable exception, there was a rough parallelism between immunosensitivity *in vitro* and *in vivo.* Immunosensitivity appeared to be related to the concentration of surface antigen, as judged by fluorescence and absorption. Immunoresistant tumors do exist that carry specific surface antigens, as measured by the fluorescence and absorption tests. This emphasizes the necessity of distinguishing between antigenicity and immunosensitivity.

The amount of infectious virus released from known numbers of irradiated cells *in vivo* appeared to be independent of the surface antigen concentration in the same tumor. These results do not critically distinguish between the possibilities that cellular antigenicity is due to the release of infectious virus and, alternatively, that a new cellular antigen appears independently of virus maturation, as in other antigenic virus-induced tumor systems.

---

[1] Abstract presented at the Conference on Murine Leukemia, Philadpphia, Penna., October 13–15, 1965. The detailed results of this work are reported in the *Journal of the National Cancer Institute,* vol. 36, 1966, pp. 607–621.

Passage of antigenic tumors in preimmunized mice did not lead to the selection of antigenic loss variants or immunoresistant sublines.

Inoculation of the Moloney agent into newborn mice leads to a state of specific immunological unresponsiveness in a certain fraction of the animals. In contrast to adults, these mice lack virus-neutralizing antibodies, or antibodies capable of reacting against Moloney lymphoma target cells in the cytotoxic or immunofluorescence tests. A second inoculation of the virus does not induce any reactivity either in these mice; this corresponds to the operational criteria of immunological tolerance. Other mice, inoculated as newborn, react with delayed or deficient antibody production.

An immunosensitive Moloney lymphoma showed better response to methotrexate therapy than an immunoresistant line. During the development of generalized lymphoma, specific antibody levels tended to disappear, but reappeared again in the course of chemotherapeutically induced regression.

DISCUSSION

Dr. Girardi: Is the immunoresistant tumor, YHA, a pure lymphoma or part lymphosarcoma? If you have a mixture of YHA cells and serum not showing the cytotoxic response, does implanting the mixture *in vivo* result in enhancement of tumor growth?

Dr. Klein: On the first question: I don't know. We have not tried to differentiate these lymphomas morphologically.

On the second question: Yes, it is possible to get enhancement if a fairly large serum dose is added to a moderate dose of cells and the mixture is inoculated into syngeneic hosts. The serum-cell ratio is very critical.

Dr. Salaman: We have been studying the immunodepressive effect in mice of Friend, Moloney, and several other viruses, and some results are possibly relevant to what you told us just now about the return of antibody.

Friend virus inoculated as late as 2 days before sheep red cells profoundly depresses production of hemolysin to the sheep red cells. This has been observed before. But it also depresses a lot of other immune reactions during the preleukemic period. On the other hand, Moloney virus has no effect on immune reactions to sheep red cells throughout the preleukemic period, whereas when the mice do become leukemic, about 50% have depressed reactions, the rest have normal reactions. So the two diseases are entirely different in this respect.

Erythropoietic Response of Animals Infected With Various Strains of Friend Virus [1]

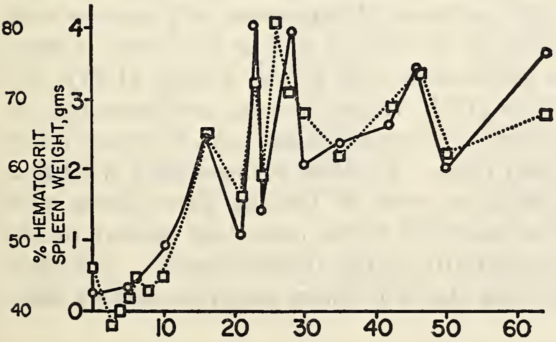
Edwin A. Mirand, *Roswell Park Memorial Institute and the State University of New York at Buffalo, Buffalo, New York*

Many investigators have shown various pathological expressions of Friend's leukemia among different strains of mice and rats (*1–8*). Few have stressed the erythropoietic response in the Friend virus disease. Mirand (*9*, *10*) was the first to report the hypervolemic polycythemia observed in this disease. Others have not seen or have not paid much attention to this phase of the disease. For this reason, we would like to emphasize some of our studies in this area.

In our early studies, we observed a marked increase in hematocrit in adult mice at about 20 days after infection. Elevation of hematocrit was not seen but, rather, anemia was reported in studies by Friend (*1*), Metcalf *et al.* (*2*), and others (*3–7*). Analysis of the data from infected Ha/ICR Swiss, DBA/1, and DBA/2 mice (*9*) revealed an initial drop in hematocrit level in the first few days after infection; however, after 3 days there was a steady rise, and after the 3d week it was common for us to see hematocrit levels at 60, 70, 80, and even 90% (text-fig. 1 and table 1). Variation in hematocrit levels was related directly to splenomegaly (text-fig. 1). There was good correlation of the hematocrit and hemo-

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.



Text-figure 1.—Increase in spleen weight ○, and hematocrit values □ in Swiss mice at intervals (days) following inoculation with Friend virus (10^{-1}).

TABLE 1.—Representative blood counts from Friend-virus-infected Ha/ICR mice

Mouse number	Days post infection	Spleen weight (g)	RBC 10^6	Hematocrit	Hemoglobin (g/100 cc)	% erythroblasts (number/200 nucleated cells)	WBC 10^3
A. Infected mice							
1	19	2.20	9.37	55	16.6	0	78.5
2	19	2.66	10.96	61	19.9	0	128.0
3	22	3.46	12.41	74	18.8	2.5	117.0
4	22	2.68	11.82	74	19.0	0	83.0
5	22	2.95	10.13	76	21.4	1.0	72.0
6	22	0.15	7.82	46	15.0	0	11.2
7	26	0.43	10.30	59	18.2	0	12.5
8	26	1.81	9.53	66	18.8	1.0	70.2
9	26	2.20	10.23	65	19.5	1.5	10.2
10	26	1.53	8.39	51	15.0	0	22.8
11	40	4.80	16.08	86	25.4	13.5	136.1
12	41	3.67	17.58	81	22.0	12.0	88.5
Average		2.38	11.21	66	19.1	2.6	69.2
B. Noninfected mice							
1	0	0.16	8.0	45	14.2	0	2.8
2	0	0.16	7.3	43	15.1	0	7.2
3	0	0.14	7.9	42	13.8	0	5.5
Average		0.15	7.7	43	14.4	0	5.2

globin levels and erythrocyte count, as shown in table 1. A progressive rise in plasma volume, as determined by I^{131}-labeled albumin (RISA), accompanied the increase in erythroid elements. At 7 days post infection the plasma volume had increased 2.5%, at 21 days 28.3%, and at 45 days 48%. Total red cell mass, as determined by Cr^{51}-labeling of RBC, increased 158% at 21 days and 491% at 45 days post infection. Determination of red-cell survival time with Cr^{51} indicated that red cell destruction was within the normal range. Therefore, the polycythemia observed in Friend virus disease is not attributed to delayed destruction of the red blood cells.

The question arose as to whether the persistent hypervolemic polycythemia observed by us was peculiar, for some reason, to the strains of mice we were using, since the pathological expression of a murine viral leukemia depends on variables of the host as well as the virus. Consequently, joint studies were undertaken with Dr. C. Friend (1961), Dr. Moloney (1964), and Dr. Rich (1965) to compare the erythropoietic response in mice from their respective laboratories and ours following infection with each strain of Friend virus. The data are presented in tables 2 through 7. The erythropoietic response of Taconic Farm Swiss mice (Dr. Friend's strain) and of Ha/ICR Swiss mice (our strain) to Dr. Friend's strain of virus was essentially similar (tables 2 and 3). BALB/c mice (Dr. Moloney's strain) and Ha/ICR Swiss mice (our strain) demonstrated the polycythemic response following infection with the Moloney strain of Friend virus; however, the response was not as marked (tables 4 and 5). We can conclude from these results that Friend's, Moloney's, and Mirand's strains of Friend virus produced some differences in the erythropoietic response, but these are only quantitative. The Rich strain of Friend virus, however, did not produce an increase in hematocrit values or an increase in the red blood cell (RBC) count in his Swiss mice or ours (tables 6 and 7); rather, an anemia was produced in our mice. The variations observed in response to infection with the various strains of Friend virus cannot be attributed to dose of virus administered. All animals received 0.2 ml intraperitoneally of a filtrate prepared from pooled 10% spleen homogenate and contained $10^{3.1}$ to $10^{3.8}$ ID50 per ml of virus. The fact that the various strains of virus gave different responses in the same strain, Ha/ICR Swiss, tends to support the idea that we may be dealing with variants of Friend virus, or that there are two closely related strains of virus, one of which induces polycythemia and is absent in the Rich filtrate.

To further understand the erythropoietic response in the Friend virus disease, radio-iron absorptions were carried out (*9*). A marked selective increase in Fe^{59} uptake by the spleen was observed. This increase in Fe^{59} uptake preceded the onset of the clinical manifestations of the disease. In contrast to the spleen, other organs showed no significant increase in Fe^{59} uptake (tables 8 and 9). The 24-hour Fe^{59} uptake was related to spleen size (text-fig. 2). The increase in Fe^{59} uptake began very early and preceded splenic enlargement, leveled off at about the time the spleen reached maximum size, and as the disease progressed there was a decrease

TABLE 2.—Friend virus (Friend strain) in Taconic Swiss mice

Days post infection	Virus-infected		
	Average RBC 10^6	Average hematocrit	Average spleen weight (g)
7	9. 69 (7. 3–10. 6)	52 (46–60)	0. 38 (0. 29–0. 55)
15	10. 46 (8. 5–12. 3)	56 (49–63)	3. 17 (2. 8–3. 7)
20	11. 34 (10. 7–13. 9)	68 (63–76)	3. 49 (2. 3–3. 9)
28	13. 11 (12. 0–14. 0)	71 (68–76)	2. 42 (1. 3–3. 09)
36	13. 20 (10. 5–14. 8)	71 (63–76)	3. 46 (3. 3–3. 6)
43	11. 70 (10. 7–12. 7)	70 (62–77)	3. 34 (2. 3–4. 4)
49	13. 0 (11. 0–15. 0)	71 (65–76)	4. 42 (4. 3–4. 5)
		Noninfected	
—	9. 12 (8. 1–9. 9)	47 (45–48)	0. 23 (0. 19–0. 29)

in uptake on a spleen-weight basis. Infected splenectomized animals showed no preferential increase in Fe^{59} uptake by other organs or the bone marrow. These studies, which were later confirmed by Chamorro *et al.* (*11*), help to substantiate the unusual erythroid proliferative characteristics of this disease.

Table 3.—Friend virus (Friend strain) in Ha/ICR Swiss mice

	Virus-infected		
Days post infection	Average RBC 10^6	Average hematocrit	Average spleen weight (g)
7	9. 59	49	0. 36
	(8. 3–10. 1)	(48–53)	(0. 20–0. 67)
15	9. 20	47	1. 20
	(6. 8–11. 0)	(34–59)	(0. 34–1. 99)
20	10. 85	65	2. 85
	(7. 0–13. 0)	(49–80)	(1. 0–4. 9)
28	14. 12	81	2. 05
	(12. 7–13. 9)	(34–87)	(1. 51–2. 42)
36	14. 89	73	3. 46
	(8. 4–18. 3)	(43–87)	(0. 39–4. 62)
43	13. 48	68	2. 56
	(8. 7–18. 8)	(45–83)	(0. 19–4. 0)
		Noninfected	
—	8. 73	47	0. 18
	(7. 1–9. 4)	(43–49)	(0. 17–0. 19)

Table 4.—Friend virus (Moloney strain) in BALB/c mice

	Virus-infected		
Days post infection	Average RBC 10^6	Average hematocrit	Average spleen weight (g)
7	10. 1	48	0. 19
	(9. 2–11. 2)	(44–52)	(0. 11–0. 28)
14	8. 9	49	0. 24
	(7. 9–10. 0)	(41–52)	(0. 14–0. 52)
21	9. 9	49	0. 64
	(8. 6–11. 7)	(45–55)	(0. 30–0. 92)
28	11. 7	62	1. 24
	(10. 3–13. 7)	(48–75)	(0. 20–2. 52)
35	8. 8	58	0. 98
	(6. 7– 9. 4)	(48–74)	(0. 13–3. 25)
42	11. 1	52	0. 15
	(10. 0–12. 8)	(50–56)	(0. 11–0. 36)
49	12. 0	57	0. 59
	(10. 4–16. 4)	(52–73)	(0. 12–1. 40)
56	8. 5	51	0. 12
	(5. 4–14. 4)	(49–52)	(0. 11–0. 13)
84	9. 4	49	0. 11
	(7. 9–10. 0)	(48–53)	(0. 09–0. 15)
		Noninfected	
—	10. 1	51	0. 11
	(8. 8–12. 3)	(49–54)	(0. 09–0. 16)

The spleen is essential for the abnormal erythroid proliferation in this disease (*10*, *12*). Removal of this organ suppressed the development of polycythemia (text-figs. 3 and 4) and erythroblastosis and even significantly delayed the onset of leukocytosis. However, it did not prevent the development of the fatal reticulum cell disease. Removal of the spleen from mice, which had already developed the full-blown Friend virus disease syndrome, produced a striking fall in hematocrit and erythrocyte levels as well as leukocyte levels (text-fig. 5). Although the spleen was responsible for the enhanced erythropoietic and early leukocytic responses, there was evidence that the liver is important in the later development of

TABLE 5.—Friend virus (Moloney strain) in Ha/ICR Swiss mice

	Virus-infected		
Days post infection	Average RBC 10^6	Average hematocrit	Average spleen weight (g)
7	8. 2 (7. 8–8. 9)	41 (39–45)	0. 30 (0. 14–0. 45)
14	8. 8 (7. 9–9. 6)	50 (43–58)	1. 23 (0. 89–1. 84)
21	10. 3 (8. 3–13. 8)	57 (41–81)	1. 81 (0. 96–2. 35)
35	10. 8 (8. 5–13. 5)	60 (43–75)	1. 90 (0. 89–2. 80)
		Noninfected	
—	8. 6 (8. 1–9. 3)	46 (44–47)	0. 11 (0. 09–0. 13)

TABLE 6.—Friend virus (Rich strain) in Swiss mice

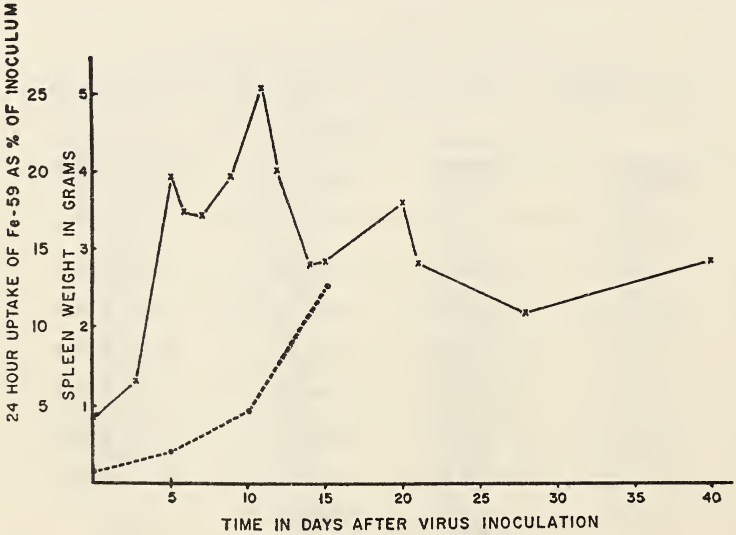
	Virus-infected		
Days post infection	Average RBC 10^6	Average hematocrit	Average spleen weight (g)
7	7. 7 (7. 2–8. 5)	45 (42–46)	0. 21 (0. 18–0. 22)
14	7. 5 (6. 1–8. 7)	46 (40–52)	0. 82 (0. 25–1. 53)
21	8. 7 (7. 3–10. 0)	44 (42–46)	0. 85 (0. 51–1. 10)
35	9. 8 (8. 9–10. 8)	44 (41–47)	0. 71 (0. 25–1. 75)
49	8. 2 (7. 1–9. 0)	45 (39–47)	0. 38 (0. 21–1. 00)
70	9. 1 (8. 1–9. 7)	45 (42–47)	0. 52 (0. 18–1. 02)
		Noninfected	
—	8. 6 (6. 9–9. 6)	48 (44–53)	0. 17 (0. 11–0. 30)

TABLE 7.—Friend virus (Rich strain) in Ha/ICR Swiss mice

	Virus-infected		
Days post infection	Average RBC 10^6	Average hematocrit	Average spleen weight (g)
7	8. 8 (8. 3–9. 2)	44 (41–48)	0. 19 (0. 16–0. 24)
14	6. 6 (5. 8–7. 6)	38 (34–40)	1. 01 (0. 34–2. 41)
21	6. 7 (5. 8–7. 5)	37 (33–40)	2. 62 (1. 75–4. 01)
35	5. 9 (5. 7–6. 1)	34 (30–38)	2. 02 (1. 42–3. 01)
		Noninfected	
—	8. 9 (8. 0–9. 1)	46 (43–48)	0. 12 (0. 09–0. 14)

leukocytosis as well as the progressive anemia that develops in the absence of the spleen (text-fig. 6). Histological examination of large livers showed massive infiltration of reticulum cells, a few erythropoietic foci, and many foci of mature cells of the granulocytic series. There was no evidence of bone marrow hyperplasia even in the presence of pronounced anemia, and, in many instances, the bones marrow actually appeared depressed.

Although the polycythemic response can be prevented by splenectomy, it can be re-established in splenectomized mice by subcutaneous transplan-



TEXT-FIGURE 2.—Mass of spleen, ●, and 24-hour uptake of Fe^{59}, X, in Swiss mice infected with Friend virus (10^{-1}).

TABLE 8.—Twenty-four-hour uptake* of Fe^{59} and hematocrit in Ha/ICR Swiss mice at weekly intervals following infection with Friend virus (10^{-1})

	7 days	14 days	21 days	28 days	Controls†
Blood	21. 1 ±4. 5	28. 1 ± 3. 4	25. 3 ± 6. 3	25. 9±4. 5	26. 9±3. 7
Liver	18. 6 ±3. 9	13. 0 ± 6. 4	8. 1 ± 1. 8	10. 8±5. 0	11. 3±2. 9
Spleen	17. 6 ±2. 8	12. 3 ± 4. 4	13. 8 ± 5. 8	11. 1±1. 5	3. 0±0. 67
Kidney	1. 0 ±0. 9	1. 1 ± 0. 3	0. 9 ± 0. 22	1. 1±0. 2	0. 8±0. 2
Femur	0. 11±0. 4	0. 11± 0. 1	0. 15± 0. 13	0. 2±0. 1	0. 3±0. 16
Hematocrit	39. 5 ±3. 3	57. 4 ±11. 3	63. 6 ±11. 8	71. 1±8. 1	43. 5±1. 9

*Uptake in 24 hours expressed as percent of inoculum.

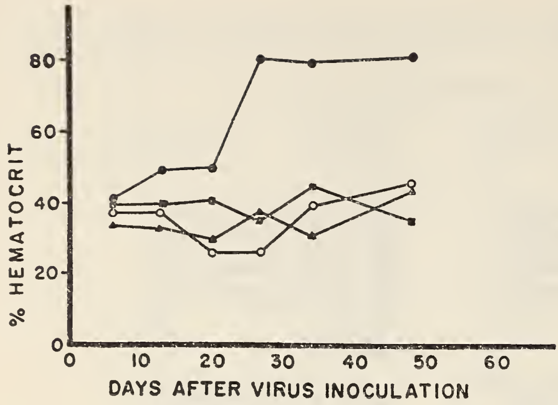
†Controls at 7 days are shown. These results are essentially identical with those at subsequent times.

TABLE 9.—Twenty-four-hour uptake* of Fe^{59} and hematocrit in DBA/1 mice at weekly intervals following infection with Friend virus (10^{-1})

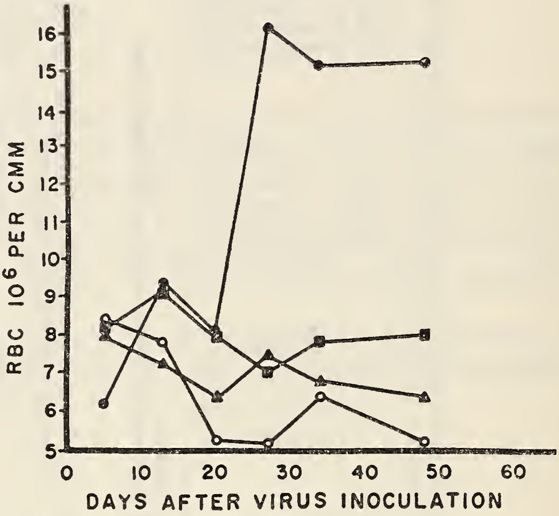
	7 days	14 days	21 days	28 days	Controls†
Blood	24. 0±6. 1	26. 1±2. 1	25. 9±1. 2	25. 1±6. 2	27. 2±1. 4
Liver	14. 5±2. 1	15. 1±1. 8	12. 1±2. 4	15. 0±1. 3	13. 4±1. 2
Spleen	18. 5±3. 4	14. 2±1. 2	14. 8±1. 8	12. 8±4. 3	2. 8±0. 54
Kidney	1. 2±0. 8	1. 1±0. 5	1. 1±0. 13	0. 9±0. 23	1. 2±0. 8
Femur	0. 11±0. 05	0. 13±0. 08	0. 14±0. 09	0. 18±0. 09	0. 3±0. 09
Hematocrit	37. 8±1. 2	62. 3±8. 5	60. 3±9. 5	74. 8±7. 2	41. 5±1. 2

*Uptake in 24 hours expressed as percent of inoculum.

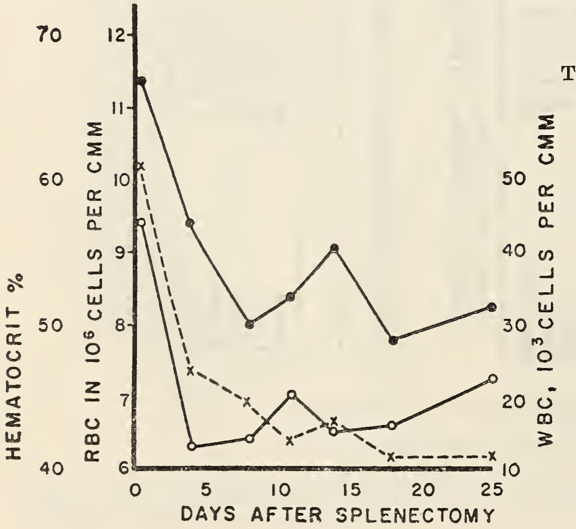
†Controls at 7 days are shown. These results are essentially identical with those at subsequent times.



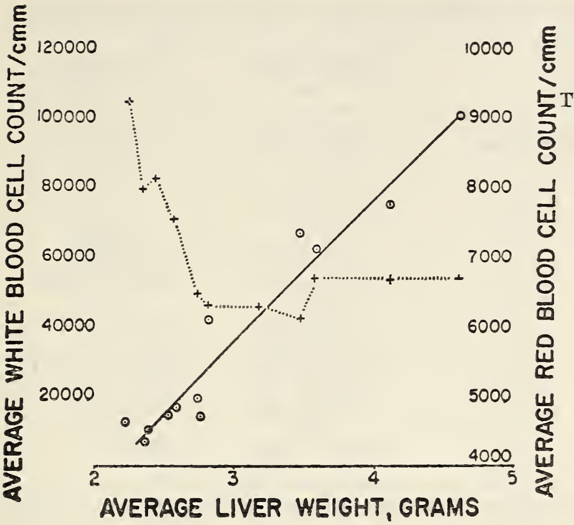
TEXT-FIGURE 3.—Effect of splenectomy on development of polycythemia in Swiss mice infected with Friend virus (10^{-1}). ● Virus-infected nonsplenectomized mice; ■ uninfected splenectomized mice; ▲ virus-infected mice splenectomized 3 days prior to virus inoculation; ○ virus-infected mice splenectomized 4 days after virus inoculation.



TEXT-FIGURE 4.—Effect of splenectomy on development of polycythemia in Swiss mice infected with Friend virus (10^{-1}). ● Virus-infected nonsplenectomized mice; ■ uninfected splenectomized mice; ▲ virus-infected mice splenectomized 3 days prior to virus inoculation; ○ virus-infected mice splenectomized 4 days after virus inoculation.



TEXT-FIGURE 5.—Effect of splenectomy on full-blown Friend virus disease in Swiss mice; virus was inoculated 21 days prior to splenectomy. ● RBC; X, hematocrit; ○ WBC.



TEXT-FIGURE 6.—Relationship between hepatomegaly, leukocytosis, and anemia in splenectomized mice receiving Friend virus; animals were splenectomized 3 days prior to virus inoculation. +, RBC; ⊙ WBC.

tation of isologous spleen (*12*). Splenectomized DBA/1 mice bearing isologous spleen transplants were infected with Friend virus. The polycythemic response was restored, although it did not occur as early as it does in intact infected mice; hematocrit levels in the 60 to 71% range were not observed until about 60 days post infection.

One can conclude, then, that splenectomy prevents the abnormal erythropoietic response and delays the development of leukocytosis, but has no effect on development of the reticulum cell leukemia.

Adrenalectomy and thymectomy, regardless of the time when performed, did not alter the response of Ha/ICR Swiss, DBA/1, and DBA/2 mice to Friend virus.

Friend virus disease was originally considered to have a narrow and specific murine host range. However, we have found that it was possible to produce lymphatic leukemia, preceded by anemia, in Sprague-Dawley rats by inoculating Friend virus into conventional (*7*) and germfree newborn and 2- to 7-day-old rats. The incidence of leukemia was fairly high, and splenomegaly and hepatomegaly were common (table 10).

When cell-free filtrates from the spleen, liver, or thymus of these rats were inoculated into Ha/ICR Swiss mice, typical Friend leukemia and polycythemia developed (table 11). Friend virus, like Moloney (*13*), Gross (*14*), Graffi (*15*), Rauscher (*16*), and Buffett-Grace-Mirand (*17*) viruses, can transcend the species barrier and produce leukemia in the rat. It is interesting, however, that the five leukemia viruses listed above produce a similar disease in both rat and mouse. Friend virus, on the other hand, produces a distinctly different disease in the mouse and rat. Whether this is a host response to the same virus or whether the Friend filtrate might have contained a lymphatic leukemia virus still has to be determined.

TABLE 10.—Leukemia induced in newborn Sprague-Dawley rats by Friend virus

Parameter*	Virus-infected rats	Noninfected rats
Number of leukemias/Number infected	158/263	0/20
Percent incidence	60. 7	0
Average time to death (days)	130 (104–163)	—
Hematocrit	26. 2 (9–41)	50 (48–52)
RBC (10^6/mm^3)	4. 084 (1. 08–6. 9)	9. 925 (9. 860–9. 990)
WBC (10^3/mm^3)	99. 9 (4. 5–722)	12. 8 (11. 5–14. 2)
Liver (g)	14. 26 (7. 63–39. 47)	14. 47 (11. 56–17. 38)
Spleen (g)	3. 57 (1. 19–9. 73)	0. 62 (0. 53–0. 71)
Thymus (g)	1. 02 (0. 10–11. 00)	0. 67 (0. 64–0. 70)

*RBC, red blood cells; WBC, white blood cells; hematocrit (HT) measured as percentage. Organ values are based on wet weights.

Vertical transmission of Friend virus disease from infected mothers to offspring was demonstrated in our laboratory (*18*, *19*). A difference was observed in the erythropoietic response between young mice inoculated directly with the virus and offspring naturally infected from their mothers. The naturally infected offspring developed a pronounced and progressive anemia (table 12). Subsequent passages of splenic filtrate from these mice produced the characteristic reticulum cell disease, as originally reported by Dr. Friend, in 90% of infected newborn and young adult mice, but polycythemia was not evident. The reasons for this difference in the pattern of host reactivity are not clear, although several possibilities are suggested:

1) Concentration of the virus was not sufficient to elicit the polycythemic response.
2) Transmission of the disease by natural means resulted in selection of a Friend virus variant that does not possess the ability to stimulate an erythropoietic response even though its ability to produce the reticulum cell disease is retained.
3) Our original inoculum pool contained more than one agent. In this instance, one might speculate that one agent is responsible for the polycythemia and that only the agent responsible for the reticulum cell disease was transmitted from mother to offspring.

We have attempted to isolate more than one agent from Friend virus disease by inoculating mouse-embryo cell cultures with Friend virus filtrates which give us routinely a polycythemic or anemic response (*20*, *21*). However, we have not succeeded in separating a "reticulum cell virus" and a "polycythemia virus"; the tissue culture filtrates gave us the same response in mice as the original filtrate used to inoculate the tissue culture preparation (tables 13 and 14).

TABLE 11.—Ha/ICR Swiss mice given injections of leukemia rat splenic filtrate*

Number positive									Percent blood differential		
									Myeloid series		
Number infected	Percent incidence	Average time to death (days)	Average liver weight (g)	Average spleen weight (g)	Average thymus weight (g)	Average RBC 10^6	Average hematocrit	Average WBC 10^3	Immature	Mature	Lymphocytes
					A. Infected						
24/30	80	34 (22–47)	3.10 (1. 87–6. 32)	2.20 (0. 97–3. 37)	0.09 (0. 06–0. 13)	10.2 (3. 8–15. 7)	54† (15–80)	68.4 (10–281)	32 7–66	38 17–70	30 18–46
					B. Noninfected						
10	— —	— —	2. 00 (1. 6–2. 5)	0. 14 (0. 10–16)	0. 08 (0. 06–0. 10)	7. 25 (7–8. 5)	43 (42–46)	4. 7 (3. 8–9. 5)	2 0. 4	23 16–30	70 56–78

*0.2 ml of a 10% w/v mince of leukemic rat spleen in Locke-Ringer's solution.

†Many of the mice demonstrated an erythropoietic response as reported by Mirand *et al.* (*9*).

TABLE 12.—Occurrence of Friend virus disease (FVD) in 309 offspring from infected Ha/ICR Swiss mothers

Group	Occurrence	Average body weight (g)	Average spleen weight (g)	Average liver weight (g)	Average RBC 10^6	Average hematocrit	Average WBC 10^3	Average time of death (due to FVD) (days)
Offspring of infected mothers developing disease	22 (7. 1%)	35 (28–44)	1. 54 (0. 3–4. 5)	5. 17 (2. 4–7. 5)	2. 47 (1. 39–2. 75)	14 (12–16)	214 (61–473)	212 (51–373)
Offspring of infected mothers free of disease	287 (92. 9%)	39* (35–45)	0. 13 (0. 10–0. 16)	2. 2 (1. 9–2. 5)	9. 05 (8. 59–9. 5)	43 (39–45)	8. 75 (6. 1–9. 0)	—

*Average values of only resistant offspring of infected mothers are indicated.

TABLE 13.—Polycythemic response of Ha/ICR Swiss mice to filtrate from tissue culture inoculated with Friend virus (Mirand strain)

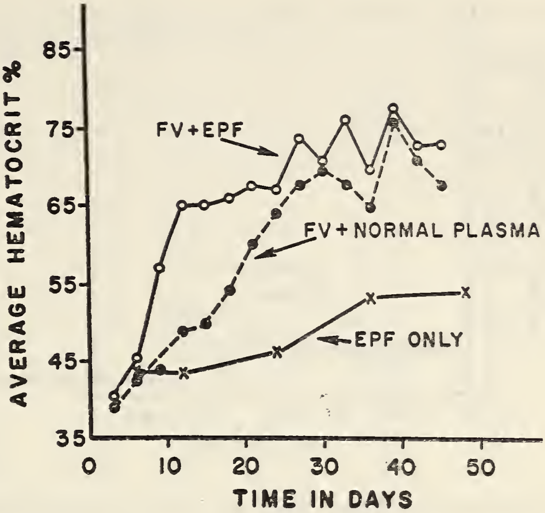
Filtrate	Number of polycythemic mice / Number of mice inoculated	Percent incidence	Average time to death
(A) from infected spleen	95/114	83. 3	55 (16–234)
(B) from tissue culture	102/122	83. 6	55 (12–218)

The problem of the physiological control of erythropoiesis is complex (*22*, *23*). In recent years it has been believed that erythropoietin (EPF) acts upon stem cells, stimulating them to differentiate into the erythrocytic series (*24*). In mice, the same type of cell immediately under the splenic capsule appears to be stimulated by EPF as well as by Friend virus. For this reason, we wanted to determine the role of EPF in Friend virus disease. Therefore, EPF obtained from animals made anemic by repeated bleeding and/or administration of phenylhydrazine (*25*, *26*) was administered intraperitoneally for 3 days prior to injection of Friend virus and daily thereafter for 48 days. It was noticed that the polycythemic response, as judged by increase in hematocrit level, occurred earlier in mice receiving EPF and Friend virus and was pronounced at 10 days rather than at the usual 20 days (text-fig. 7) (*27*, *28*). Also, the spleens of mice given EPF plus Friend virus were consistently larger than those of mice given normal plasma and Friend virus (text-fig. 8). More intense erythropoietic activity in the spleens of the former group was confirmed histologically. Thus, EPF plus Friend virus had an additive effect in erythropoietic stimulation as long as the spleen was intact. However, splenectomized mice receiving EPF plus Friend virus did not develop polycythemia (text-fig. 9).

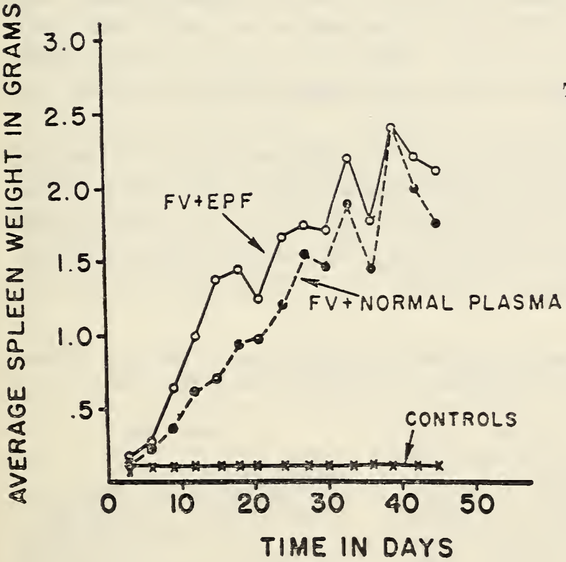
A relationship is seen between splenomegaly and inhibition of erythropoiesis in the bone marrow of Ha/ICR Swiss mice inoculated with Friend virus (table 15). This was determined not only by study of bone marrow smears but also by measurement of 24-hour Fe^{59} uptake by the bone

TABLE 14.—Anemic response to filtrate from tissue culture inoculated with Friend virus obtained from offspring of mothers infected with Friend virus (Mirand strain)

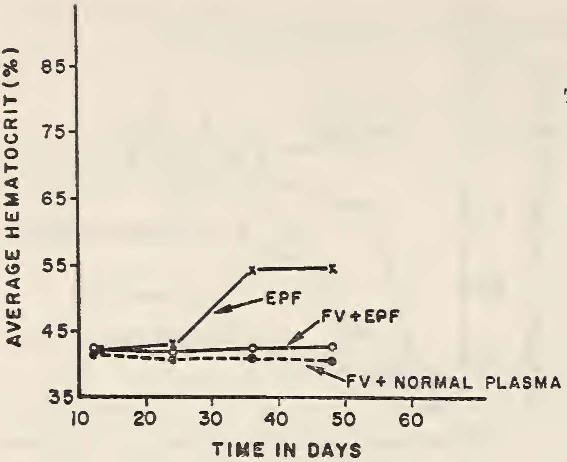
Filtrate	Number of anemic mice / Number of mice inoculated	Percent incidence	Average time to death
(A) from infected spleen	45/57	79. 0	39 (9–189)
(B) from tissue culture	56/65	86. 2	42 (10–168)



TEXT-FIGURE 7.—Effect of Friend virus and erythropoietin on hematocrit values in Ha/ICR Swiss mice.



TEXT-FIGURE 8.—Effect of Friend virus and erythropoietin on spleen weight of Ha/ICR Swiss mice. Controls received erythropoietin alone.



TEXT-FIGURE 9.—Effect of Friend virus and erythropoietin on hematocrit values in splenectomized Ha/ICR Swiss mice.

marrow of infected mice at various intervals after virus infection. One can speculate that the spleen produces a factor which inhibits erythropoiesis in the bone marrow (*29*). Attempts to isolate this "factor" from large Friend-virus-infected spleens have not yet been successful (*30*).

Response of hypertransfused-polycythemic (HP) mouse to Friend and Rauscher viruses.—EPF production and erythropoiesis are virtually eliminated in a hypertransfused-induced polycythemia produced by repeated intraperitoneal injections of washed RBC (*31–33*). This is illustrated by the low Fe^{59} uptakes in the bone marrow (femur), blood, and spleen of hypertransfused-polycythemic animals as compared with those in untreated control mice (table 16). EPF is the only factor reported to date that can break through the hypertransfused-polycythemic state and re-establish erythropoiesis.

In view of the marked erythropoietic stimulus provided by Friend and Rauscher viruses, we were interested in determining whether these viruses were capable of re-establishing erythropoiesis in the hypertransfused-polycythemic state. Hypertransfused-polycythemic mice were given various strains of Friend virus and Rauscher virus. They were given 0.5 mc

TABLE 15.—Relationship between inhibition of erythropoiesis in the bone marrow and splenomegaly in Ha/ICR Swiss mice infected with Friend virus

	Normal		Friend virus	
Days post infection	Percent 24-hour Fe^{59} uptake—bone marrow	Spleen weight (g)	Percent 24-hour Fe^{59} uptake—bone marrow	Spleen weight (g)
7	0. 50	0. 09	0. 43	0. 14
10	0. 42	0. 09	0. 24	1. 35
14	0. 40	0. 14	0. 10	2. 37
21	0. 43	0. 09	0. 08	3. 79
28	0. 50	0. 08	0. 09	2. 91

TABLE 16.—Response of hypertransfused-polycythemic (HP) Ha/ICR Swiss mice to Friend virus (Mirand strain)

Group	Days post infection	Average 24-hour Fe^{59} uptake* Femur	Blood	Spleen	Average spleen weight* (g)	Average hematocrit*
(A) Untreated control	7	0. 50	24. 7	2. 0	0. 11	42
Friend virus	7	0. 43	25. 7	4. 2	0. 75	43
HP	7	0. 15	0. 3	0. 4	0. 12	71
HP + Friend virus	7	1. 9	1. 9	1. 1	0. 18	74
(B) Untreated control	14	0. 40	25. 9	1. 5	0. 12	43
Friend virus	14	0. 10	26. 8	17. 9	1. 10	51
HP	14	0. 10	0. 3	0. 5	0. 14	75
HP + Friend virus	14	0. 10	18. 2	12. 1	0. 58	73
(C) Untreated control	28	0. 48	29. 9	1. 8	0. 15	42
Friend virus	28	0. 08	27. 0	13. 7	2. 38	68
HP	28	0. 10	0. 3	0. 6	0. 12	71
HP + Friend virus	28	0. 09	29. 3	14. 5	1. 06	79

*Average from minimum of 5 mice per group.

of Fe^{59} intravenously at various intervals following infection, and Fe^{59} uptake by the femur, blood, and spleen was determined 24 hours later.

Friend virus (*Mirand strain*).—There was evidence of the re-establishment of erythropoiesis as early as 7 days after infection in these mice. Fe^{59} uptake by the blood and spleen increased markedly at 14 and 28 days, but it is interesting to note that the Fe^{59} uptake by the bone marrow was decreased at 14 and 28 days after infection as splenomegaly developed (table 16).

Friend virus (*Moloney's strain*).—This strain of virus also contains a polycythemic component as evidenced by the fact that it can re-establish erythropoiesis in the hypertransfused-polycythemic state (table 17). However, the response was not as marked as that observed in hypertransfused-polycythemic mice given Mirand strain of Friend virus.

Friend virus (*Rich's strain*).—Whereas the Mirand and Moloney strains of Friend virus evidenced a polycythemic component and were capable

TABLE 17.—Response of hypertransfused-polycythemic (HP) Ha/ICR Swiss mice to Friend virus (Moloney strain)

Group*	Average 24-hour Fe^{59} uptake† Femur	Blood	Spleen	Average spleen weight (g)†	Average hematocrit†
Untreated control	0. 48	27. 5	1. 3	0. 14	44
Friend virus	0. 18	24. 8	8. 4	0. 95	57
HP	0. 09	0. 2	0. 1	0. 12	72
HP + Friend virus	0. 07	5. 9	3. 8	0. 64	78

*All data obtained 14 days after infection with Friend virus (Moloney).
†Average from minimum of 5 mice per group.

of re-establishing erythropoiesis in a hypertransfused-polycythemic mouse, the Rich strain of Friend virus did not (table 18).

Rauscher virus.—Like the Rich strain of Friend virus, this virus did not re-establish erythropoiesis in the hypertransfused-polycythemic mouse (table 19). Moreover, there appears to have been a marked inhibition of development of the characteristic splenomegaly and reticulum-erythroid proliferation which was evident as late as 28 days post infection. Curiously, there was inhibition of erythropoiesis in the bone marrow even though gross splenomegaly was not evident. Apparently, even mild splenomegaly can reduce erythropoiesis in the bone marrow (table 19).

In table 20, a comparison of response of hypertransfused-polycythemic Ha/ICR Swiss mice to various strains of Friend virus and Rauscher virus at 14 days post infection is illustrated. Using the hypertransfused-

TABLE 18.—Response of hypertransfused-polycythemic (HP) Ha/ICR Swiss mice to Friend virus (Rich strain)

Group*	Average 24-hour Fe^{59} uptake†			Average spleen weight (g)†	Average hematocrit†
	Femur	Blood	Spleen		
Untreated control	0. 55	25. 8	1. 7	0. 15	42
Friend virus	0. 25	27. 4	7. 8	0. 89	37
HP	0. 08	0. 3	0. 09	0. 14	76
HP + Friend virus	0. 10	0. 5	0. 14	0. 29	74

*All data obtained 14 days after infection with Friend virus (Rich).

†Average from minimum of 5 mice per group.

TABLE 19.—Response of hypertransfused-polycythemic (HP) Ha/ICR Swiss mice to Rauscher virus

Group	Days post infection	Average 24-hour Fe^{59} uptake*			Average spleen weight* (g)	Average hematocrit*
		Femur	Blood	Spleen		
(A) Untreated control	7	0. 46	25. 2	1. 3	0. 11	42
Rauscher virus	7	0. 48	34. 6	6. 6	0. 38	39
HP	7	0. 12	0. 2	0. 5	0. 13	74
HP + Rauscher virus	7	0. 49	0. 7	0. 9	0. 29	72
(B) Untreated control	14	0. 51	28. 2	2. 1	0. 15	42
Rauscher virus	14	0. 30	25. 0	15. 5	1. 13	40
HP	14	0. 08	0. 3	0. 2	0. 11	78
HP + Rauscher virus	14	0. 17	0. 4	0. 5	0. 41	73
(C) Untreated control	20	0. 43	26. 4	1. 2	0. 14	45
Rauscher virus	20	0. 24	18. 5	20. 6	1. 78	40
HP	20	0. 09	0. 4	0. 2	0. 10	71
HP + Rauscher virus	20	0. 18	0. 6	0. 2	0. 39	75
(D) Untreated control	28	0. 47	25. 3	1. 8	0. 13	43
Rauscher virus	28	0. 27	19. 8	10. 0	1. 89	35
HP	28	0. 08	0. 2	0. 1	0. 12	72
HP + Rauscher virus	28	0. 17	0. 6	1. 2	0. 29	67

*Average from minimum of 5 mice per group.

TABLE 20.—Comparison of response of hypertransfused-polycythemic Ha/ICR Swiss mice to various strains of Friend virus and Rauscher virus*

Virus strain	Average 24-hour Fe^{59} uptake†			Average spleen weight (g)†	Average hematocrit†
	Femur	Blood	Spleen		
Friend virus (Mirand)	0. 10	18. 2	12. 1	0. 58	73
Friend virus (Moloney)	0. 07	5. 9	3. 8	0. 64	78
Friend virus (Rich)	0. 10	0. 5	0. 14	0. 29	74
Rauscher virus	0. 17	0. 4	0. 5	0. 41	73

*All data collected 14 days after infection with virus.
†Average from minimum of 5 mice per group.

polycythemic mouse, it appears, then, that one can distinguish on a functional basis among the various strains of Friend virus having a polycythemic component (table 20). Also, the data show that the Rich strain of Friend virus and the Rauscher virus share an inability to re-establish erythropoiesis in the hypertransfused-polycythemic state.

The mechanism by which Friend virus can re-establish erythropoiesis in the hypertransfused-polycythemic state is of utmost importance to our comprehension of the process of erythropoiesis. As stated previously, erythropoietin is the only factor now known that can release the inhibition of erythropoiesis in the hypertransfused-polycythemic state. Also, it has been established (*24*) that erythropoietin acts upon the stem cell or primitive mesenchyme, inducing cellular differentiation to the erythroid series. Indeed, there is much evidence that erythropoietin may be the physiological regulator of erythropoiesis (*34–36*). The question arises as to whether Friend virus produces its polycythemia in susceptible mice, or reestablishes erythropoiesis in a hypertransfused-polycythemic state by excessively stimulating cells that produce erythropoietin which initiates erythropoiesis, or whether it is capable of initiating erythropoiesis without erythropoietin. Perhaps the treatment of noninfected and infected spleen cells in tissue culture with erythropoietin will answer this fundamental question.

DISCUSSION

In summary,

1) Friend virus (Mirand) produced a hypervolemic polycythemia in susceptible mice. This activity was present in Friend virus supplied by Dr. Friend and Dr. Moloney but not in Dr. Rich's strain of Friend virus.

2) The polycythemia observed was spleen-dependent. Splenectomy inhibited the polycythemia and delayed the leukocytosis until hepatomegaly developed in virus-infected animals. Splenectomy did not prevent the development of reticulum cell leukemia. Subcutaneous isologous spleen transplants restored the polycythemic response.

3) Friend virus induced lymphatic leukemia and anemia in Sprague-Dawley rats; however, filtrates from leukemic rats returned to susceptible mice induced the characteristic polycythemia and reticulum cell leukemia.

4) Offspring from mothers infected with Friend virus did not develop polycythemia but did develop the characteristic reticulum cell disease. Also, filtrates from such offspring did not induce the polycythemic response but did induce the reticulum cell disease as originally reported by Dr. Friend.

5) There was a relationship between the degree of polycythemia and of splenomegaly in mice receiving Friend virus. Also, there was a relationship between the inhibition of erythropoiesis in the bone marrow and splenomegaly. Whether this was due to a direct action of the virus on the bone marrow or to a splenic bone marrow inhibitory factor produced by the infected spleen is not known.

6) Exogenous erythropoietin accelerated the appearance of polycythemia in Friend-virus-infected mice and did not produce polycythemia in splenectomized infected mice.

7) The Mirand and Moloney strains of Friend virus were capable of re-establishing erythropoiesis in hypertransfused-polycythemic mice, but the Rich strain of Friend virus and the Rauscher virus did not re-establish erythropoiesis. With the hypertransfused-polycythemic mouse, the polycythemic component in a Friend virus filtrate can be functionally determined and analyzed.

8) Friend virus, like erythropoietin, can release the inhibition of erythropoiesis during a hypertransfused-polycythemic state. Whether Friend virus releases this inhibition by excessively stimulating cells to produce erythropoietin, or through an alternative mechanism independent of erythropoietin cannot be answered at this time.

9) The possibility that Friend virus disease may be due to more than one related virus has been considered; however, attempts to prove this have not been successful. Even splenic tissue culture preparations infected with Friend virus (Mirand strain) still give the biphasic response—polycythemia and reticulum cell proliferation.

10) We believe Friend virus disease to be an excellent model for the study of erythropoiesis and for the further elaboration of the action of erythropoietin.

SUMMARY

Various strains of Friend virus gave different erythropoietic responses. All strains of virus produced reticulum cell proliferation and erythroblastosis, but in addition, some, particularly the Mirand strain, produced a characteristic hypervolemic polycythemia. This was spleen-dependent; splenectomy prevented development of hypervolemic polycythemia in virus-infected animals. However, the polycythemic response was not prevented in the infected, splenectomized animal bearing a subcutaneous

isologous spleen transplant. Offspring from infected mothers developed reticulum cell proliferation and erythroblastosis but not hypervolemic polycythemia. Rats infected with one strain of Friend virus developed lymphatic leukemia and anemia. When rat-passaged Friend virus was returned to mice, Friend virus disease with hypervolemic polycythemia was observed. Exogenous erythropoietin accelerated the appearance of polycythemia in Friend-virus-infected mice but not in splenectomized mice. Friend virus (Mirand strain), like erythropoietin, released the inhibition of erythropoiesis produced during the hypertransfused-polycythemic state. Whether Friend virus released this inhibition by stimulating cells to produce erythropoietin or by some other mechanism independent of erythropoietin cannot be answered at this time. These studies made it possible to distinguish among the various strains of Friend virus on the basis of polycythemia in the normal animal. This was further substantiated by the response in the hypertransfused state.

REFERENCES

(*1*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307–318, 1957.

(*2*) Metcalf, D., Furth, J., and Buffett, R. F.: Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res 19: 52–58, 1959.

(*3*) Fieldsteel, A. H., Dawson, P. J., and Bostick, W. L.: Quantitative aspects of Friend leukemia virus in various murine hosts. Proc Soc Exp Biol Med 108: 826–829, 1961.

(*4*) Dawson, P. J., Fieldsteel, A. H., and Bostick, W. L.: Pathogenic studies of Friend virus leukemia and the development of a transplantable tumor in BALB/c mice. Cancer Res 23: 349–354, 1963.

(*5*) Ludwig, F. C., Bostick, W. L., and Epling, M. L.: Quantitative analysis of Friend's disease in two inbred strains of mice with emphasis on bone marrow response. Cancer Res 24: 1308–1317, 1964.

(*6*) Tsuchida, R., and Rich, M. A.: Chromosomal aberrations in viral leukemogenesis. I. Friend and Rauscher leukemia. J Nat Cancer Inst 33: 33–47, 1964.

(*7*) Mirand, E. A., and Grace, J. T., Jr.: Induction of leukemia in rats with Friend virus. Virology 17: 364–366, 1962.

(*8*) Siegler, R., and Rich, M. A.: Comparative pathogenesis of murine viral lymphoma. Cancer Res 24: 1406–1417, 1964.

(*9*) Mirand, E. A., Prentice, T. C., Hoffman, J. G., and Grace, J. T., Jr.: Effect of Friend virus in Swiss and DBA/1 mice on Fe^{59} uptake. Proc Soc Exp Biol Med 106: 423–426, 1961.

(*10*) Mirand, E. A., Hoffman, J. G., Grace, J. T., Jr., and Trudel, P. J.: Modification of the Friend virus disease by splenectomy. Proc Soc Exp Biol Med 107: 824–828, 1961.

(*11*) Chamorro, A., Latarjet, R., Vigier, P., and Zajdela, F.: New investigations on the Friend disease. *In* Ciba Found Symp Tumor Viruses of Murine Origin. Boston, Little, Brown & Co., 1962, pp 176–184.

(*12*) Mirand, E. A.: Further evidence that the spleen is necessary to produce the erythropoietic syndrome in Friend virus disease. Amer Zool 1: 212, 1961.

(*13*) Moloney, J. B.: Properties of a leukemia virus. Nat Cancer Inst Monogr 4: 7–37, 1960.

(*14*) Gross, L.: Induction of leukemia in rats with mouse leukemia (passage A) virus. Proc Soc Exp Biol Med 106: 890–893, 1961.

(*15*) Graffi, A., and Gimmey, J. Z.: Uber die Wirkung der myeloischen leukemie der Maus bei dev Ratte. Ges inn Med m ihre grenzgebiete 13: 881–884, 1958.

(*16*) Rauscher, F. J.: Pathogenesis of a murine stem-cell leukemia induced by a newly isolated virus. Acta Un Int Cancr 19: 368–373, 1963.

(*17*) Buffett, R. F., Grace, J. T., Jr., DiBerardino, L. A., and Mirand, E. A.: Recovery of murine leukemia virus prior to development of leukemia growth curve and dose-response. Cancer Res. In press.

(*18*) Mirand, E. A., and Grace, J. T., Jr.: Transmission of Friend virus disease from infected mothers to offspring. Virology 16: 344–347, 1962.

(*19*) Mirand, E. A.: Transmission of Friend virus through mother's milk. *In* Viruses, Nucleic Acids, and Cancer. Baltimore, Williams & Wilkins Co., 1963, pp 146–147.

(*20*) Osato, T., Mirand, E. A., and Grace, J. T., Jr.: Propagation and immunofluorescent investigations of Friend virus in tissue culture. Nature (London) 201: 52–54, 1964.

(*21*) ———: Unpublished data.

(*22*) Gordon, A. S.: Hemopoietine. Physiol Rev 39: 1–40, 1959.

(*23*) Mirand, E. A., Gordon, A. S., and Wenig, J.: Mechanism of testosterone action in erythropoiesis. Nature (London) 206: 270–272, 1965.

(*24*) Lajtha, L. G.: Stem cell kinetics and erythropoietin. *In* Erythropoiesis (Jacobson, L. O., and Doyle, M., eds.). New York, Grune & Stratton, 1962, pp 140–150.

(*25*) Mirand, E. A., Prentice, T. C., and Slaunwhite, W. R.: Current studies on the role of erythropoietin on erythropoiesis. Ann NY Acad Sci 77: 677–702, 1959.

(*26*) Mirand, E. A., Prentice, T. C., and Grace, J. T., Jr.: Ability of tamarins to produce and respond to erythropoietin. Nature (London) 204: 1064–1066, 1964.

(*27*) Mirand, E. A., and Grace, J. T., Jr.: The effect of erythropoietin on the erythropoietic response in the Friend virus disease. Proc Amer Assoc Cancer Res 4: 44, 1963.

(*28*) ———: Some current findings in Friend leukemia. Accad Nazionale dei Lincei, Quaderno N 65: 9–31, 1964.

(*29*) Dameshek, W.: Hypersplenism. Bull NY Acad Med 31: 113–136, 1955.

(*30*) Mirand, E. A.: Unpublished data.

(*31*) Mirand, E. A., and Prentice, T. C.: Fe^{59} assessment of erythropoiesis in mice following injections of anemic rabbit plasma extracts. Proc Soc Exp Biol Med 95: 164–167, 1957.

(*32*) Fried, W., Plzak, L. F., Jacobson, L. O., and Goldwasser, E.: Studies on erythropoiesis. III. Factors controlling erythrocyte production. Proc Soc Exp Biol Med 94: 237–241, 1957.

(*33*) Filmanowicz, E., and Gurney, C. W.: Studies on erythropoiesis. XVI. The response to a single dose of erythropoietin in the polycythemic mouse. J Lab Clin Med 57: 65–72, 1961.

(*34*) Bethell, F. H., Linman, J. W., and Korst, D. R.: Erythropoietic activity on anemic and polycythemic plasmas. Trans Assoc Amer Physicians 70: 297–304, 1957.

(*35*) Gurney, C. W., Goldwasser, E., and Pan, C.: Studies on erythropoiesis. VI. Erythropoietin in human plasma. J Lab Clin Med 50: 534–542, 1957.

(*36*) Mirand, E. A., Weintraub, A. H., Gordon, A. S., Prentice, T. C., and Grace, J. T., Jr.: Erythropoietic activity of untreated and deproteinized normal human plasma. Proc Soc Exp Biol Med 118: 823–826, 1965.

DISCUSSION

Dr. Sinkovics: Did anybody look at the juxtaglomerular cells of the kidney with the electron microscope to see whether these viruses, Friend or Rauscher, would set up a focus of multiplication there and would suppress or increase (by causing proliferation of these cells) erythropoietin production there?

Dr. Mirand: We haven't done that. This area has been considered as a possible site in the kidney for erythropoietin production. For this reason, I think the kidney lesion observed is significant and might play an important role in the erythropoietic response in the Friend disease. As you know, the kidney has been proposed as being the major site for erythropoietin production, but it is not the sole site for erythropoietin production. Evidence for this was established initially by us (Proc. Soc. Exp. Biol. Med. 96 : 49–51, 1957 ; Ann. N.Y. Acad. Sci. 77 : 677–702, 1959) and confirmed by others several years ago.

Erythrocytic Maturation *In Vitro* of Murine (Friend) Virus-Induced Leukemic Cells [1, 2]

C. FRIEND, M. C. PATULEIA, and E. DE HARVEN,[3]
Sloan-Kettering Institute for Cancer Research, New York, New York

THE question of whether leukemic cells possess the mechanism of maturation is an intriguing one (*1*, *2*). The reports that such phenomena have been observed *in vitro* (*3*, *4*) have been met with some skepticism, due primarily to the fact that they stemmed from short-term tissue culture studies and had not been adequately confirmed. Nevertheless, in examining the extensive work that has been done on the culture of normal and abnormal blood cells (*5*, *6*), the claims that maturation has been observed come up too frequently to be ignored. The findings in the present report indicate that some leukemic cells grown *in vitro* exhibit a limited capacity to mature along the erythrocytic line.

In a previous study (*7*), it was shown that subcutaneous implantation of spleen or liver fragments from DBA/2 mice in advanced stages of virus-induced leukemia resulted in tumor formation at the site of inoculation in recipient mice of the same inbred strain. These tumors, resembling reticulum cell sarcomas, were transplantable and have been maintained in serial passage.

Tissue culture lines have been established from a number of these tumors. The cells adapted to culture readily, growing in suspension, and have retained their tumor-producing properties to date, a year after the initiation of the cultures. Examinations of stained preparations of the tissue culture leukemic cells revealed the presence of cells maturing along the erythrocytic series. Evidence that these cells may be elaborating hemoglobin is suggested by an increased uptake of Fe^{59} and the presence of benzidine-positive material.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported by Public Health Service research grants CA-02062 and CA-08067 from the National Cancer Institute, Gen. Res. Support Grant NIH 1 SO1 FR 05534, and NYC Health Res. Council Contracts U-1096 II C2 and I-325.

[3] We are indebted to Dr. Lloyd Old and Miss Elizabeth Stockert for performing the cytotoxic tests; to Dr. Mary Jane Hamilton and Miss Mary Ruth for the absorption spectra and the benzidine tests. The technical assistance of Miss Ingrid Kuechenthal, Mr. Gilbert Holland, and Mr. Archibald Kentigan is gratefully acknowledged.

MATERIAL AND METHODS

Tumors.—Of the 7 tumor lines that have been followed during the past few years, all have contained appreciable amounts of virus for varying periods during their transplantation history. Generally at about the level of the 30th serial transplant passage, a decrease in virus was noted. In some cases, no infectious virus was recovered when the material was assayed in adult Swiss mice by routine titration procedures (*8*). When, however, extracts of the spleen from the tumor-bearing animals were passaged in newborn mice, virus was generally demonstrable. Therefore, these tumors were not considered to be virus-free, and tissue culture lines were established in the hope that such a system might shed some light on the virus-host-cell relationship.

The tumors used in this study had been in serial passage in DBA/2 mice for 62 generations or more. The mice were sacrificed 3 to 4 weeks after the subcutaneous implantation by trocar of tumor fragments, and the tumor was aseptically removed.

Tissue culture.—The organ culture technique of Jensen *et al.* (*9*) was used to initiate the cultures. The tumor was cut into explants 1 to 2 mm in size. Two fragments were placed on each tea-bag paper disc supported on a stainless-steel mesh platform in a 60 × 15 mm petri dish containing 10 ml of medium. This method was uniformly successful, for it permitted individual cells to migrate through the mesh paper into the medium. The cells grew vigorously in suspension and were transferred for maintenance to prescription bottles as soon as the increase in cell volume warranted it. The original explants continued to shed viable cells for periods exceeding 2 months.

Dehydrated basal medium (Eagle) with Earle's balanced salt solution (Grand Island Biological Company), supplemented with 10% unheated fetal calf or agamma bovine serum, was used. The medium was changed twice weekly. The petri dish cultures were incubated at 37° C in a continuous stream of water-saturated 5% CO_2 in air. The bottles were kept in incubators to which no CO_2 was supplied.

For routine passage, approximately 1×10^5 cells per ml were added to 32-ounce prescription bottles containing 60 ml of medium. The cells more than doubled in number within 24 hours. Cell counts were done with trypan blue added to estimate the ratio of living cells to dead cells. The cultures were routinely checked for the presence of pleuropneumonia-like organisms (PPLO) on PPLO medium plates supplied by Hyland Laboratories, Los Angeles, California.

Stained preparation.—Two ml aliquots of tissue culture fluid containing the cells in suspension were spun in a refrigerated International centrifuge at approximately 1000 rpm for 10 minutes. As much of the supernatant fluid as possible was removed without disturbing the cell pellet. One drop of serum was added to the tube, and the cells were gently resuspended. One drop of the mixture was placed on a slide and smeared as is done with blood. The films were air-dried and stained with Wright's or Leishman's.

Mice.—DBA/2 mice, obtained from The Jackson Laboratory, Bar Harbor, Maine, were used to assay the tissue-cultured cells for tumor production. Swiss mice (ICR/Ha) purchased from Millerton Farms, Millerton, New York, were used for the titration of virus in the tissue-cultured cells or supernatant fluid.

Fe^{59} uptake studies.—Ferrutope—radio-ferrous (Fe^{59}) citrate—was obtained from E. R. Squibb & Sons, New York, New York. Counting was done on a Nuclear Chicago Gamma Ray Spectrometer.

Sixty ml of medium in 32-ounce prescription bottles was seeded so that each ml contained about 1×10^5 cells per ml. Experiments were conducted with cultures to which Fe^{59} in 1, 2, and 5 μc amounts was added. At daily intervals, 2 ml for a vital cell count and 10 ml for isotope assay were removed from each culture. The bottles were gassed with CO_2 before being returned to the incubator. The cell suspensions for isotope assay were centrifuged about 3000 rpm for 15 minutes. The supernatant was removed carefully so as not to disturb the cells, and a 2 ml aliquot of the supernatant was retained for counting. The cells were washed 4 times in normal saline; a 2 ml aliquot of the last washing was also kept for counting. The cells, brought up to 2 ml with distilled water containing a small amount of saponin to enhance lysis, were then assayed for isotope.

Test for benzidine-positive material.—Samples of tissue culture fluid containing about 4 million cells each were washed 3 times in Ringer's solution. They were then resuspended in 2 cc of distilled water to which $MgCl_2$ to a final concentration of 0.005 M had been added to enhance lysis. After low-speed centrifugation to remove cell debris, the supernatant fluid was analyzed for the presence of benzidine-positive material according to the method of Shinowara and Walters (*10*). Absorption spectra were carried out on aliquots of the same extracts with the Beckman DU spectrophotometer.

RESULTS

Cell Morphology

In examining stained preparations of the tissue-cultured cells, at least two cell types may be observed (figs. 1, 2, 3, and 4). Both types may show cytoplasmic vacuoles similar to those observed in Burkitt's lymphoma cells (*11*, *12*). This is especially apparent when the cells are in need of a media change. The best preserved cell appeared to be a proerythroblast, for the cytoplasm stained a deep blue and the nucleus was hyperchromatic with course chromatin clumps. Occasionally these cells contained more than one nucleus. Erythroblasts in more advanced stages of maturity were also present, although reticulocytes or fully mature anucleate erythrocytes have not as yet been seen. The other cell type appeared to be less differentiated and probably represents a primitive reticulum cell. It was somewhat larger, and both its nucleus and cytoplasm were paler staining than the first cell type. The chromatin was arranged in a fine filigree pattern and nucleoli were more prominent than those found in the proerythroblast. These cells were more fragile than the erythroblasts, since the smears frequently contained numbers of pale smudged cells difficult to identify positively. Mitosis was frequently observed among both the proerythroblasts and reticulum cells.

To date, 11 lines of cells have been established from individual leukemic tumors. All have grown readily in suspension and have shown similar morphology, although they may vary in regard to the ratio of stem cells to erythroblasts.

Effect of Sera

The cells propagate equally well in media containing either 10% agamma or fetal calf serum. However, it is our impression that the percentage of erythroblasts in the culture grown in fetal calf is higher than in those grown in agamma calf serum. That these differences may be attributable to varying amounts of iron present in the sera is now under investigation.

Calf serum supports the cells adequately but growth is slower. This is an adventure, since, with those lines maintained in calf serum, media changes are made but once a week.

Rate of Growth

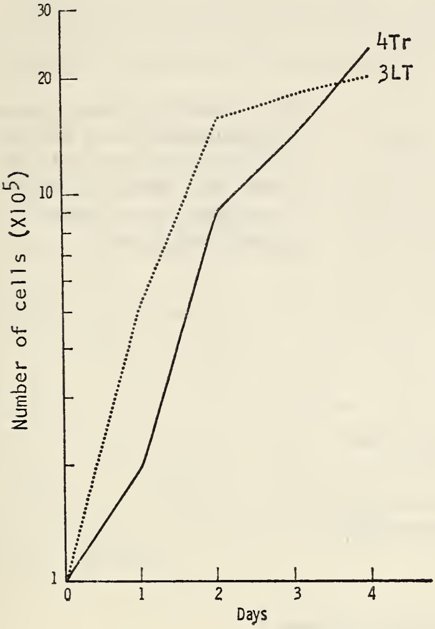
The growth curves for 5 of the leukemic cell lines are shown in text-figures 1 and 2. All doubled in number within 24 hours. The two oldest lines (3LT and 4Tr), which were initiated in October, 1964, appear to have a doubling time of about 14 hours (text-fig. 1). The other 3, which are in continuous cultures for from 3 to 7 months, exhibit similar doubling times (text-fig. 2).

Fe^{59} Incorporation

Since chromatophilic erythroblasts, although few, were observed in these cultures, it was of interest to determine whether hemoglobin was being synthesized.

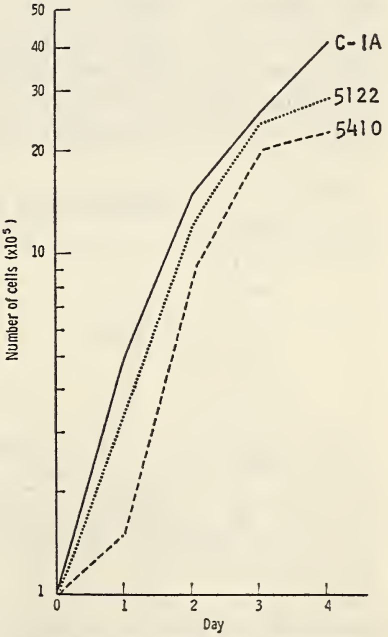
Experiments were set up with the SKL-1 line of human leukemic cells as control cultures. This line, derived from the cells of a patient with lymphoblastic leukemia, was obtained from Dr. Bayard Clarkson.

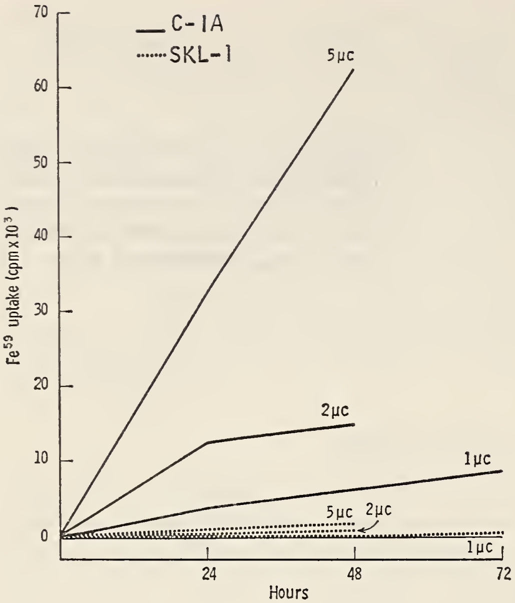
Fe^{59} (sp. act. 13.7 μc/mg) in 1, 2, and 5 μc amounts was added to the SKL-1 and our C-IA cultures. The results are illustrated in text-figure 3. Whereas no difference in uptake was observed in the SKL-1 cultures, the uptake in the C-IA cultures was marked, indicating that the iron was being incorporated.



Text-figure 1.—Growth rates of tissue culture lines designated 3LT and 4Tr in continuous culture since October, 1964. Both demonstrate a doubling time of approximately 14 hours.

Text-figure 2.—Growth rates of tissue culture lines designated C-IA, 5122, and 5410, which have been in continuous culture for from 3 to 7 months. The rates are comparable to those observed in text-figure 1.

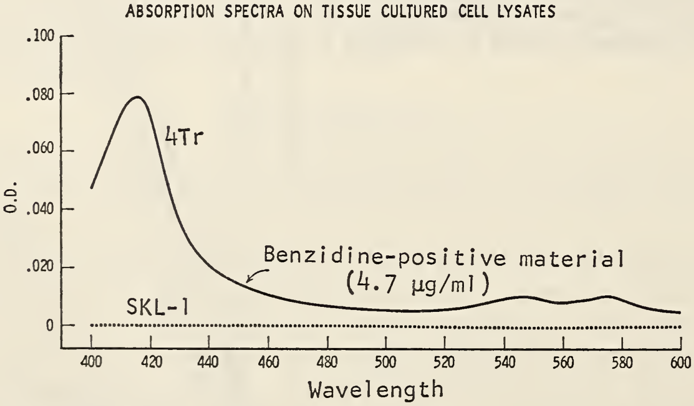




TEXT-FIGURE 3.—Comparison of the incorporation of Fe^{59} into leukemic cell lines C-IA (murine) and SKL-1 (human). Iron added to the media in 1, 2, and 5 μc amounts.

Benzidine-Positive Material

With the SKL-1 leukemic cell line used once again as control, extracts of the cells were examined for benzidine-positive material. At the same time, absorption spectra of the cell extracts were determined as shown in text-figure 4. The cell extract of the murine leukemic line, established for 13 months and designated 4Tr, contained 4.7 μg per ml of benzidine-positive material, in contrast to the cell extract of the human SKL-1 line which contained undetectable amounts.



TEXT-FIGURE 4.—Spectrum of the extract of leukemic cell from line 4Tr (murine) compared with that of line SKL-1 (human). The 4Tr extract contained 4.7 μg per ml of benzidine-positive material.

Although the benzidine-positive material could be an indication of the presence of peroxidase, the absorption spectrum of the same material suggests that it may be hemoglobin.

Assay for Tumor Production

The leukemic cell lines are sensitive to Friend and Rauscher cytotoxic antisera and produce tumors upon implantation into DBA/2 mice (fig. 5), the strain in which the tumors originated. As few as 500 cells will produce large tumors within 3 weeks after inoculation. These are histologically indistinguishable from the original tumors (fig. 6), which were used as the starting material for the cultures. When these tumors are returned to tissue culture, once again, maturation along the erythrocytic line is demonstrable. Thus far, these cells do not grow when implanted into Swiss mice, although they are efficient in immunizing them against challenge with active virus preparation (*13*).

Single cell isolates are being made in the hope of determining whether the progeny of all cells capable of proliferation are malignant as well as virus producers.

Assay for Virus

As has been the experience of others working with this virus *in vitro* (*14*), little or no infectious virus can be recovered from the supernatant fluid assayed in adult Swiss mice by the usual titration methods. However, inoculation of resuspended pellets of filtered supernatant fluid into newborn mice or into the highly susceptible DBA/2 mice results in leukemia, and vaccination of Swiss mice with these pellets protects them against challenge with active virus. These data, plus the fact that electron microscope examination reveals the presence of virus particles in the supernatant fluid and budding from the membranes of the leukemic cells (*15*), indicate that small amounts of virus, some of which is incomplete perhaps, are constantly being elaborated.

DISCUSSION

There has been some difference of opinion as to the type of leukemia caused by the virus which was isolated in our laboratory, the majority of the opinions being that it is a reticulum cell sarcoma associated with erythroblastosis (*16*). Since an abnormal erythropoietic response is characteristic of the leukemia induced, questions were raised as to 1) whether the erythroblastic and neoplastic manifestations were distinct and separate processes caused by the same virus, or 2) whether there was more than one virus involved, one causing the erythroblastosis and the other the neoplastic alteration.

Zajdela (*17*) concluded from histologic and autoradiographic studies that the cells of the virus-induced leukemia, although resembling undifferentiated reticulum cells, were primitive proerythroblasts.

That certain strains of the virus may contain more than one virus is indicated by the work of Mirand (*18*, *19*) who observed hypervolemic polycythemia, rather than anemia, associated with the leukemia induced by two of the strains. The possibility that these differences may indicate a variation in host response was considered. The fact that the polycythemic effect, which accompanied infection with some strains of the leukemia virus, could be eliminated is worthy of note. If the virus was inoculated into splenectomized mice or if it was transmitted from infected mothers to their offspring, the virus induced the typical picture of anemia, erythroblastosis, and reticulum-cell proliferation (*8*, *16*), even after subsequent passage from these animals.

The response of the erythroid cells to the leukemia virus, whether manifested by anemia or polycythemia, is of particular interest in view of our recent tissue culture findings.

Our observations suggest that we are dealing with one leukemia-inducing virus. The erythropoietic response is probably an intrinsic part of this leukemia which appears to resemble the erythremic myelosis similar to that seen in di Guglielmo's disease in man (*20*).

The reticulum cell sarcomas from which the tissue culture lines are derived contain a uniform population of stem cells (fig. 6). Erythroblasts have not been observed in these transplantable tumors which originated from subcutaneous grafts of the organs from mice with virus-induced leukemia, although they are present in the leukemic infiltrates in the spleens and livers.

If, however, the reticulum cells are cultivated *in vitro*, maturation along the erythrocytic line becomes apparent. At least two types of cells emerge; the first appears to be a proerythroblast and the second a less differentiated stem cell. In addition, erythroblasts in various stages of maturation are observed. Although rare chromatophilic erythroblasts are present, no reticulocytes or fully mature erythrocytes have as yet been seen. These cells appear to carry the genetic information which permits expression of some normal function. The incorporation of iron and the presence of benzidine-positive substance suggest the possibility that hemoglobin is being synthesized. This material is being isolated and characterized.

The virus-induced leukemic cells, which have been propagating in continuous culture for a year, have retained their neoplastic properties. The tumors they produce on subcutaneous inoculation into DBA/2 mice are histologically identical to those from which the cell lines originated. They still contain the virus genome as demonstrated by infectivity and immunologic tests.

Whether the various cell types in the tissue-cultured lines are derived from one parental stem cell awaits further clarification. The analysis of the progeny of single cell isolates should provide the evidence, and such studies are in progress.

Since the Rauscher virus (*21*) also gives rise to erythroblastosis, although followed by lymphoid leukemia, it would be most interesting to determine whether the cells rendered leukemic by this virus would also exhibit the ability to mature under *in vitro* conditions.

SUMMARY

Several lines of murine leukemic cells propagating in continuous culture for a year are being investigated. The cultures were developed from the reticulum-cell sarcomas produced by subcutaneous grafting into DBA/2 mice of spleen or liver from mice with Friend virus-induced leukemia. The cells grow in suspension and appear to be maturing along the erythrocytic line. Morphologically, at least 2 cell types may be observed in stained preparations. The first resembles a proerythroblast, for the cytoplasm is deeply stained and the nucleus is hyperchromatic with coarse chromatin clumps. The other cell type is less differentiated, somewhat larger, and both its nucleus and cytoplasm are paler staining than the first cell type. The chromatin is fine and nucleoli are more prominent than those in the proerythroblast. Erythroblasts in various stages of maturity are also present. The cells retain their tumor-producing potential and continue to shed virus. The fact that they incorporate iron and contain benzidine-positive material suggests the possibility that hemoglobin is being synthesized. The significance of these findings in relation to the type of leukemia induced by the Friend virus is discussed.

ADDENDUM

It has come to our attention that, at the 1965 Tissue Culture Association Meeting, Soule *et al.* (*22*) reported erythropoietic differentiation of cells from a Stansly virus-induced leukemia of BALB/c mice observed in tissue culture.

REFERENCES

(*1*) Bessis, M.: La différenciation et la maturation des cellules leucémiques. Considérations cytologiques et cliniques. Rev Hemat 9: 745–758, 1954.

(*2*) Furth, J.: The physiopathology of leukemia. *In* The Physiopathology of Cancer (Homburger, F., ed.). New York, Hoeber-Harper, 1959, p 369.

(*3*) Awrorow, P., and Timofejewsky, A. D.: Kultivierungsversuche von leukämischen Blute. Vorsch Arch Path Anat Physiol 216: 184–214, 1914.

(*4*) Timofejewsky, A. D., and Benewolenskaya, S. W.: Neue Beobachtungen an lymphoiden Zellen der myeloiden und lymphatischen Leukämie in Explantations-Versuchen. Arch Exp Zellforsch 8: 1–34, 1929.

(*5*) Bichel, J.: On the cultivation of a mouse leukemia *in vitro*. Denmark, Univ Press of Aarhus, 1939.

(*6*) Woodlife H. J.: Blood and Bone Marrow Cell Culture. Philadelphia, J. B. Lippincott Company, 1965.

(*7*) Friend, C., and Haddad, J. R.: Tumor formation with transplants of spleen or liver from mice with virus-induced leukemia. J Nat Cancer Inst 25: 1279–1289, 1960.

(*8*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307–318, 1957.

(*9*) Jensen, F. C., Gwatkin, R. B. I., and Biggers, J. D.: A simple organ culture method which allows simultaneous isolation of specific types of cells. Exp Cell Res 34: 440–447, 1964.

(*10*) Shinowara, G. Y., and Walters, M. I.: Hematin-studies on protein complexes and determination in human plasma. Amer J Clin Path 40: 113–122, 1963.

(*11*) Wright, D. H.: Cytology and histochemistry of the Burkitt lymphoma. Brit J Cancer 17: 50–55, 1963.

(*12*) Epstein, M. A., and Barr, Y. M.: Characteristics and mode of growth of a tissue culture stain (EB1) of human lymphoblasts from Burkitt's lymphoma. J Nat Cancer Inst 34: 231–240, 1965.

(*13*) Friend, C.: Immunologic relationships among some of the murine leukemia viruses. *In* Symposia-Viral-Induced Tumors. Workshop on Prospects for Control by Immunologic Methods and Chemotherapy. In press.

(*14*) Moore, A. E.: Growth and persistance of Friend leukemia virus in tissue culture. J Nat Cancer Inst 30: 885–895, 1963.

(*15*) de Harven, E., and Friend. C.: Origin of viremia in murine leukemia. Nat Cancer Inst Monogr 22: 70–105, 1966.

(*16*) Metcalf, D., Furth, J., and Buffett, R. F.: Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res 19: 52–58, 1959.

(*17*) Zajdela, F.: Contribution à l'étude de la cellule de Friend. Bull Assoc Franc Cancer 49: 351–373, 1962.

(*18*) Mirand, E. A.: Erythropoietic response of animals infected with various strains of Friend virus. Nat Cancer Inst Monogr 22: 483–503, 1966.

(*19*) Mirand, E. A., and Grace, J. T.: Some current findings in Friend leukemia. *In* The Viruses in Leukemia of Mammals. Proc. Intern. Sympos. Nat Acad of Lincei, Rome, No. 65, pp 9–38, 1964.

(*20*) Schwartz, S. O., and Critchlow, J.: Erythremic myelosis (di Guglielmo's disease). Critical review with report of four cases and comments on erythroleukemia. Blood 7: 765–793, 1952.

(*21*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*22*) Soule, H. D., Albert, S., Wolf, P., and Stansly, P. G.: Erythropoietic differentiation of cells grown in tissue culture from virus-induced leukemia in mice. Excerpta Med 19: No. 10, 1965.

PLATES

Plate 92

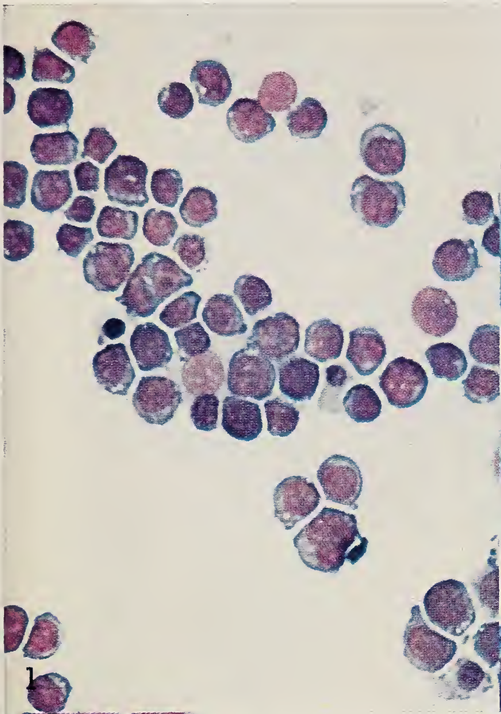
Wright's stained smear preparations of tissue-cultured virus-induced leukemic cells.

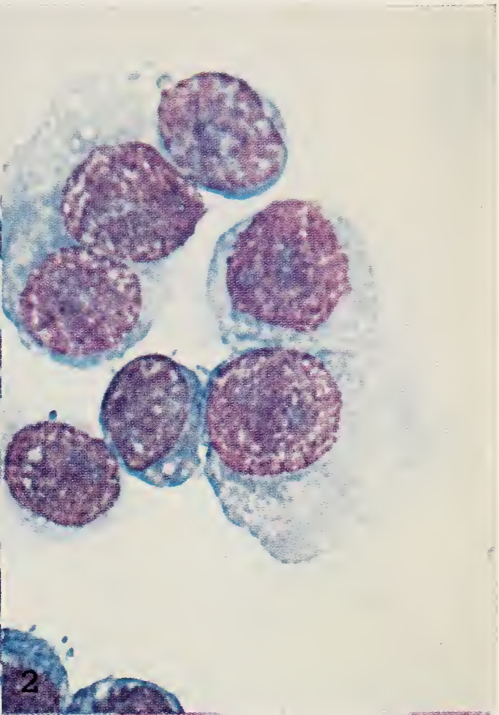
Figure 1.—Stem cells, proerythroblasts, and erythroblasts are present. × 1,040

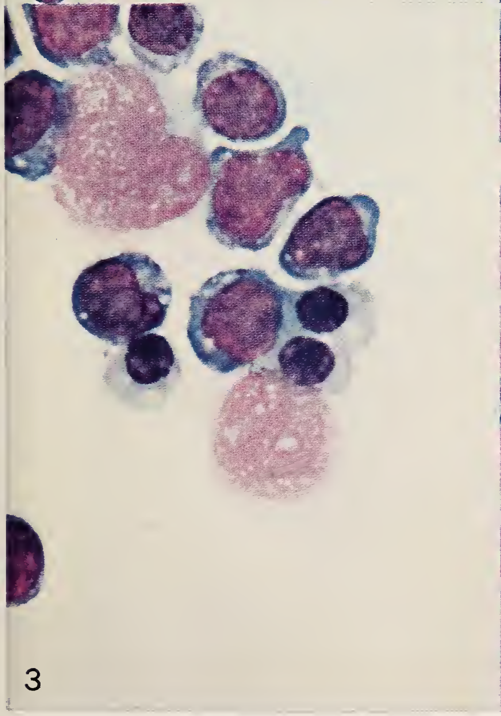
Figure 2.—Stem cells with prominent nucleoli and proerythroblasts can be seen. × 2,600

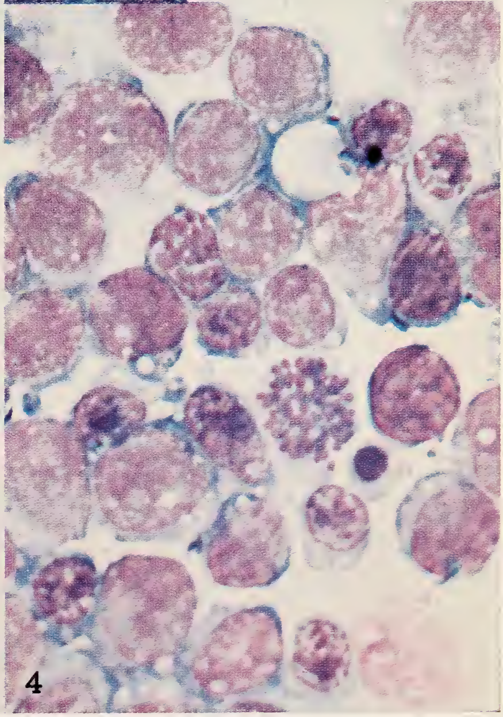
Figure 3.—Two smudged cells, presumably stem cells, proerythroblasts, and 3 basophilic erythroblasts are evident. × 2,600

Figure 4.—Cells in various stages of maturation along the erythrocytic line can be seen in this group. A mitotic figure is present. × 2,600









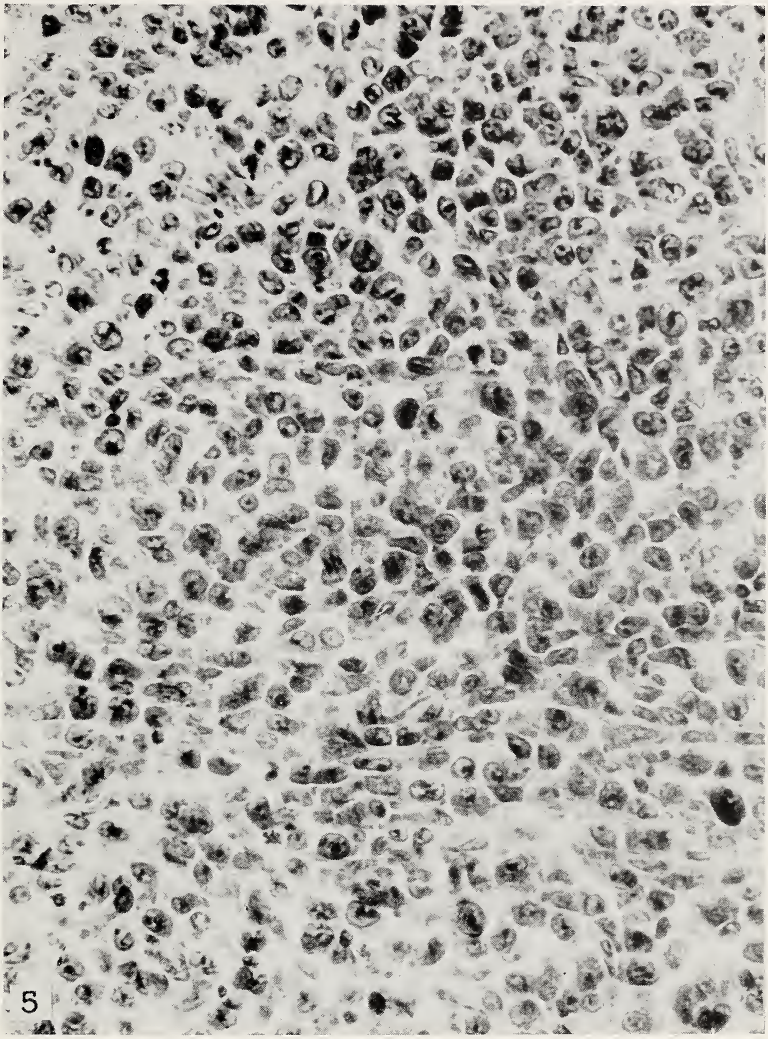
FRIEND, PATULEIA, AND DE HARVEN

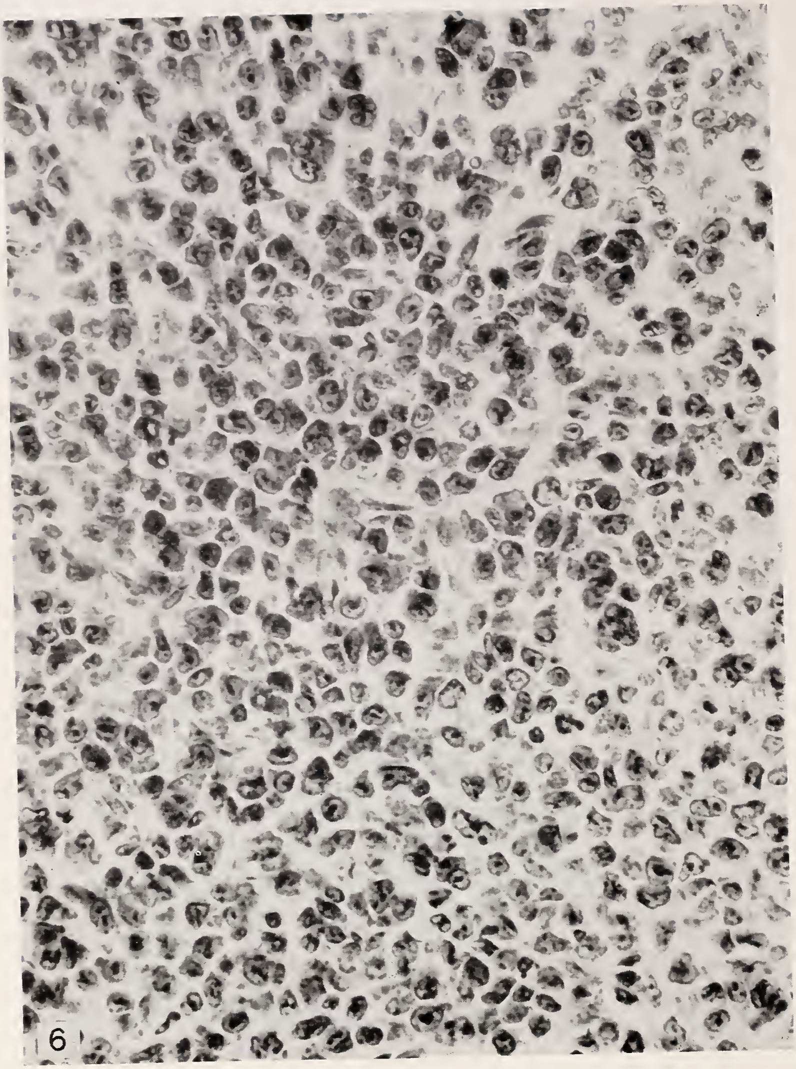
Plate 93

Sections fixed in Vandergrift's solution and stained with hematoxylin and eosin. × 250

Figure 5.—A reticulum cell sarcoma resulting from the subcutaneous inoculation of tissue-cultured leukemic cells into DBA/2 mice.

Figure 6.—A reticulum cell sarcoma from which the tissue-cultured line of leukemic cells originated. *Note* the uniform population of large immature mononuclear cells which is characteristic of this neoplasm.


5


6

DISCUSSION

Dr. Stansly: I don't know whether to consider the virus we're working with and isolated in our laboratory some years ago to be a substrain of the Gross agent or of the Friend agent. In any case, it is, as I often indicated, strongly myeloid and erythroid, and perhaps for this reason Dr. Herbert Soule in our group also could quite readily isolate cultures from tissues of neoplastic mice which differentiate into red cells *in vitro*. These cultures have now been under cultivation for over a year. This was reported at the 1965 spring meeting of the Tissue Culture Society (Excerpta Med. Sec. I, 19 No. 10, p. IV, 1965).

I have pictures demonstrating this but I don't know whether it is worthwhile or whether there is an opportunity to show them. Dr. Friend's were very beautiful and we have essentially the same type of reaction she has shown. We show hemoglobin in these cells by a staining procedure rather than by Fe^{59}. In general, we duplicate her findings very well.

These cultures produce virus and some transplant while others do not. We are isolating different cell types from these cultures by cloning, in an effort to determine which cell type is transplantable.

Dr. Block: I'd like to make a comment first on radioiron curves. The often-made assumption, that an increased uptake of radioiron over an erythropoietic organ, or even the increased appearance of radioiron in circulating red cells, indicates an increased rate of red cell formation or that the converse indicates a decreased rate of red cell formation, is erroneous. The radioiron curves may be translated into rates of red cell formation only by experienced individuals who are knowledgeable about red cell formation and who also have investigated the bone marrow, tissue iron, serum iron, and reticulocytes in the subject under study.

I'd like to address a question to Dr. Friend. I was most impressed by this important work. I notice that you showed the uptake of iron by means of a radioisotope and you also showed by means of ultraviolet-absorption spectra that the cells contained porphyrins. The absorption spectrum, if I recall correctly, shows only that the substance in the cells is a porphyrin, not hemoglobin. None of the erythroblasts had an eosinophilic cytoplasm but all had basophilic or slightly basophilic cytoplasm, indicating that these cells did not contain hemoglobin. It behooves us to ask whether these cells, although they are erythroblasts, are functioning normally. They may incorporate iron and may form protoporphyrin, but do they put iron, protoporphyrin, and globin together to form hemoglobin?

Dr. Friend: First of all, it is remarkable to find iron uptake in this *in vitro* system. These long-term tissue cultures originated from uniform populations of primitive reticulum cells and presumably no red blood cells from the original inocula could have survived over a period of a year. The absorption spectra suggest it is hemoglobin rather than porphyrin that is present. Hemoglobin peaks at 415, 540, and 575 $m\mu$, and reacts with benzidine whereas the porphyrins do not. We have yet to isolate and identify the benzidine-positive material. Although polychromatophilic and rare eosinophilic erythroblasts are observed, it is too early to state whether they are functioning normally. So far, our data indicate that they may be synthesizing hemoglobin.

Dr. Sachs: Dr. Friend, have you tried adding erythropoietin to tissue culture cells to obtain a better differentiation?

Dr. Friend: We have already started that. We have one experiment which indicates that it may be a useful method.

Dr. Stansly: Concerning the question of hemoglobin and red cells in these cultures, Dr. Albert of our laboratory has done one more thing, which is, I think, of interest in this connection, and that is to place such cultured cells in a diffusion chamber in the mouse. Under these conditions we get a significant increase in red cells inside the chamber in which we are quite sure there is no contamination from the outside, thus magnifying, so to speak, the observations we have made in the original cultures and demonstrating that differentiation is actively taking place there.

For example, you can put in such chambers tissue culture cells which already contain, say, 1,000 red cells and come out with 10 to 100 times this number of red cells after 3 days of incubation in the mouse.

Dr. Brodsky: Concerning Dr. Block's observations, we have been doing iron clearance studies in animals with Friend disease and determining serum iron and plasma iron turnovers. In the human being 90% of the injected iron (Fe^{59}) is initially taken up by the bone marrow, and certainly this appears to be the case in animals with Friend disease and in normal mice. The plasma iron turnover is the most sensitive expression of total erythropoiesis, not the uptake or the reappearance.

Under these circumstances we have not been finding an increase in the plasma iron turnover in animals infected with Friend. Our particular strain of virus, which we obtained from Dr. Friend and has been passed since 1957 in NIH Swiss Webster mice and now is being passed in Swiss mice obtained here in the Philadelphia area, persistently produces an anemia. We do not see a polycythemic phase.

We find a hemolytic anemia. The red blood cell survival as determined by the T-½ of chromium 51-labeled cells from Friend-injected animals is 6 to 8 days, whereas the chromium 51 T-½ of normal mouse red blood cells is 15 days.

Dr. Sykes: I would merely like to offer a suggestion to Dr. Friend. If there is any reasonable amount of hemoglobin present in the *in vitro* system she used, a good way of proving its presence would be by spectroscopy and the shifting of the lines following addition of carbon monoxide.

THE THYMUS IN LEUKEMOGENESIS

Chairman: Jacob Furth

TABLE 1.—Morphologic classification of the murine leukemias

	Normal cell	Neoplastic cell	Organ of origin	Designation of leukemia	Peripheral blood dissemination	Latency	Cells autonomous (transplantable)	Etiologic agents	Occurs spontaneously
"Splenic" viruses 1. Friend 2. Rauscher	Erythrocyte	Erythroblast	Spleen	Erythroblastic (Friend-Rauscher disease)	Yes	1–3 weeks	Rarely	Virus	Rarely
"Thymic" viruses 1. Gross 2. Graffi 3. Moloney 4. Rich 5. Buffett 6. Radiation leukemogenic virus*	Lymphocyte	Lymphoblast	Thymuses	Lymphocytic (malignant lymphoma)	Yes	2–3 months	Yes	Virus, X ray, chemicals, and hormones	Frequently
	Granulocyte	Myeloblast	Spleen (bone marrow)	Granulocytic (myeloblastic leukemia)	Yes	3–6 months	Yes	Virus, X ray, and chemicals	Rarely
	Reticulum cell (histiocyte?)	Reticulum sarcoma cell	Spleen nodes and bone marrow	Reticulum cell sarcoma	No	6–12 months	Rarely	Virus and chemicals	Occasionally

*Sometimes referred to as Kaplan virus (*4*).

fore should be classified along with the leukemias, even though in the reticulum cell sarcoma the neoplastic cells are not liberated into the bloodstream in large numbers.

In table 1 the major morphologic varieties of murine leukemia are listed in order of increasing latency, together with their attributes. Admittedly this table oversimplifies the complex range of morphologic varieties of tumors, and individual tumors may be found which behave contrary to the attributes listed. Study of the pathogenesis of these tumors is facilitated because they may all be induced by viral agents in mice. While the existence of other forms of murine leukemia (stem cell, monocytic, etc.) is recognized, these leukemias are omitted here for purposes of clarity and because they occur relatively infrequently.

ERYTHROLEUKEMIA

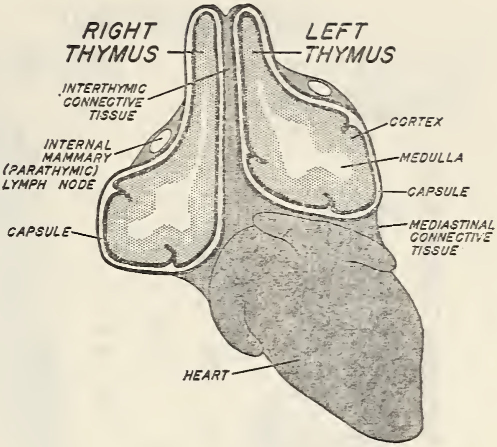
The first of the leukemias in table 1, erythroleukemia, or neoplastic proliferation of red cell precursor cells, may be exemplified by the disease produced by the Friend or Rauscher viruses, described in previous publications (*5–8*). Within a few days after inoculation of either of these viruses, the spleen begins a sustained and pronounced enlargement. Histologically, this enlargement is due to an intense proliferation of red cell precursors in the red pulp portion of the spleen (fig. 1). The proliferation of the erythroblasts in the red pulp is in marked contrast to the absence of proliferation of cells of the splenic lymphoid follicles. As the erythroblasts continue to proliferate rapidly, they are sequestered into the splenic venous sinuses (fig. 2). They pass from here into the portal vein, by which they enter the liver as free cell emboli (fig. 3). In the sinusoids of the liver, these cells may continue to proliferate or they may undergo maturation. As large numbers of these cells flood through the liver, they begin to appear in the peripheral circulation, constituting the *leukemic* phase of the disease. As these splenic erythroblasts are sequestered into the splenic venous sinuses, the architectual integrity of the splenic red pulp is destroyed. Necrosis of some cells, plus the effect of the splenic hemostatic blood pressure, creates large hemorrhagic blood pools (fig. 4). When these occur near the capsule of the spleen, splenic rupture may occur, causing death. Some animals survive, with gradual reduction in spleen size and probable maturation of erythroblasts to red cells. In some virus stocks, as reported by Dr. Mirand, a polycythemia thus results (*9*). Under other conditions where maturation does not occur, a relative anemia may result. In general, the proliferating cells of the spleen which are sequestered into the splenic veins do not continue to proliferate when they reach distant organs. In one study these leukemic cells were found to be nontransplantable (*7*), whereas transplantation has been reported in another study (*10*). This type of murine leukemia resembles the di Guglielmo syndrome of man (*11*). It has only rarely been reported to occur spontaneously in mice (*12*).

LYMPHOID LEUKEMIA

The second group of the leukemias, and the most common form of spontaneous leukemia in mice, is the lymphoid leukemia. In 1951 Dr. Gross showed that these spontaneous tumors were in fact induced by a virus (*13*). Using a comparable lymphoid leukemia virus isolated in our laboratory, we followed the tissue changes which resulted after neonatal inoculation. In our early study of the pathogenesis of this tumor, we encountered great difficulty and confusion in interpreting the histologic material because of a disparity between the histologic appearance and the total weight of what we were calling "*the thymus.*" At that time we were treating the entire thymic tissue as if it consisted of a single midline organ, "*the thymus*," which had two connected parts or "lobes." This error in our anatomical concept led us to weigh the total mass of thymic tissue rather than the individual structures. As was illustrated in previous publications dealing with the evolution of the thymic lymphoma, the two thymuses independently show the tumor change (*14–16*). By weighing the two structures together during the preneoplastic period, we were never able to quantitate these changes or to correlate weight changes with histologic changes. To clarify this problem, we made sections through the mediastinal tissue of mice, rats, guinea pigs, dogs, and man, and found that the left thymic mass and the right thymic mass were not connected. On the basis of our anatomic studies, we were able to confirm the observations of earlier anatomists which we had overlooked until then, who stated that there was no such organ as "*the thymus*," but rather that there were two paired, separate organs, the left thymus and the right thymus (*17*, *18*). The anatomic relations of the two thymuses are illustrated in text-figure 1.

Once we realized that we were dealing with two separate organs, we began to weigh each thymus separately and to correlate the histology of each thymus with its own weight. The reason this became absolutely necessary may be seen in figure 5, which illustrates the thymuses of an AKR mouse killed at 8 months of age. The right thymus, while it *appears* enlarged, is actually normal in size and weighs 57 mg, which is normal for the age and sex of this strain. The *significant* preneoplastic change has occurred in the left thymus (arrow), which weighs only 12 mg, and has undergone a marked reduction of mass. If these two thymuses had been weighed together as "the thymus," the total weight of 69 mg would not express the marked unilateral change that had occurred.

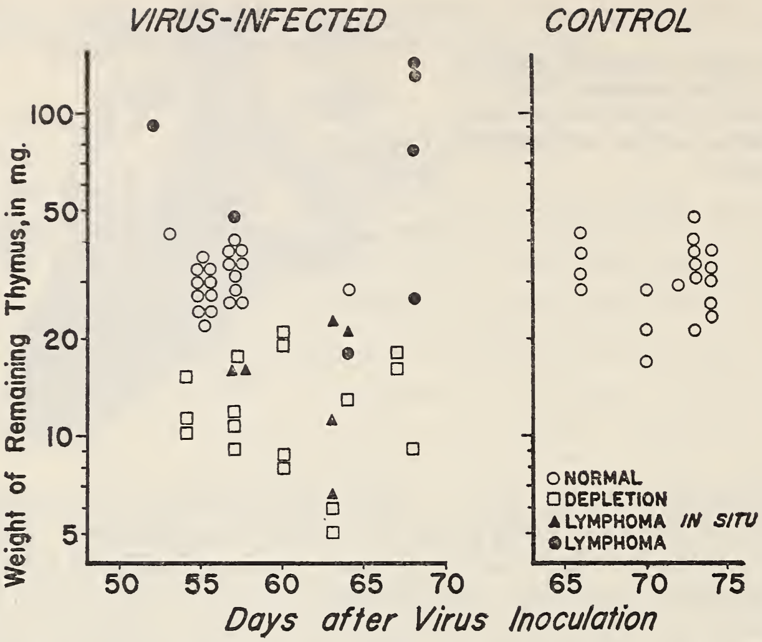
Following the demonstration that the two thymuses responded to the leukemogenic virus independently (*15*), the serial histologic changes of viral leukemogenesis were studied in unilaterally thymectomized mice.


RIGHT THYMUS
LEFT THYMUS
INTERTHYMIC CONNECTIVE TISSUE
INTERNAL MAMMARY (PARATHYMIC) LYMPH NODE
CORTEX
MEDULLA
CAPSULE
CAPSULE
MEDIASTINAL CONNECTIVE TISSUE
HEART

TEXT-FIGURE 1.—Anatomic relations of the two thymuses. Each thymus has an outer cortex, an inner medulla, and its own complete capsule.

In these mice, which had only one thymus remaining, the histologic changes could be accurately correlated with the weight changes. Since it was established in other studies (*19*) that virus-inoculated mice with only one remaining thymus developed leukemia in the same incidence and after the same latency as intact mice, the kinetics of thymic mass and the thymic histology could be assumed to reflect the actual preneoplastic changes. One such study is recorded below. Fifty-one mice were inoculated at birth with Rich leukemogenic virus (*20*). On day 5, these virus-inoculated mice and 21 uninoculated control mice were unilaterally thymectomized; equal numbers had the right or left thymus extirpated. The animals were randomly selected for sacrifice between days 55 and 68. The remaining thymus was weighed and serially sectioned for histology. The thymuses were classified into one of four histologic categories, using criteria previously reported (*15*), and the weight and diagnosis represented as a point symbol in text-figure 2. An example of the histology of the cortex of a normal, control, remaining thymus (weight 39 mg) is given in figure 6, and may be compared with the histology of a small, lymphocyte-depleted remaining thymus, in the virus-inoculated group, which weighed 6 mg (fig. 7). In figure 8, a frank lymphoma has replaced the entire thymic mass. This specimen weighed only 17 mg and was half the weight and size of a control thymus. As indicated in text-figure 2, the lymphoma *in situ* and lymphoma changes occurred in thymuses which were often smaller than normal. Once initiated, however, the lymphoma markedly enlarged the affected thymus, so that it became much heavier, as indicated by the values at the top portion of text-figure 2.

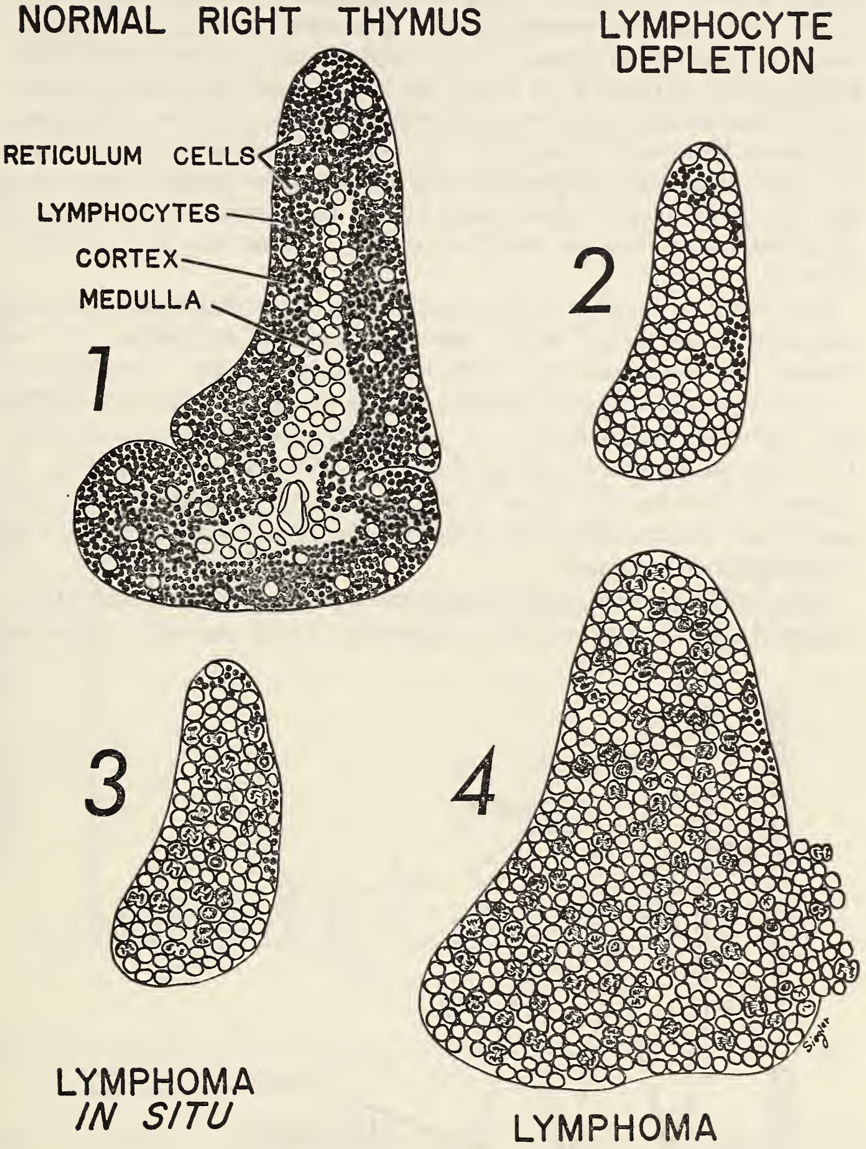
A sequence for the evolution of the lymphoma in virus-infected, unilaterally thymectomized mice is suggested in text-figure 3. This sequence fits both the subjectively derived histologic data and objectively derived weight data given in text-figure 2. This interpretation of the evolution of the lymphoma is compatible with the results and interpretations of



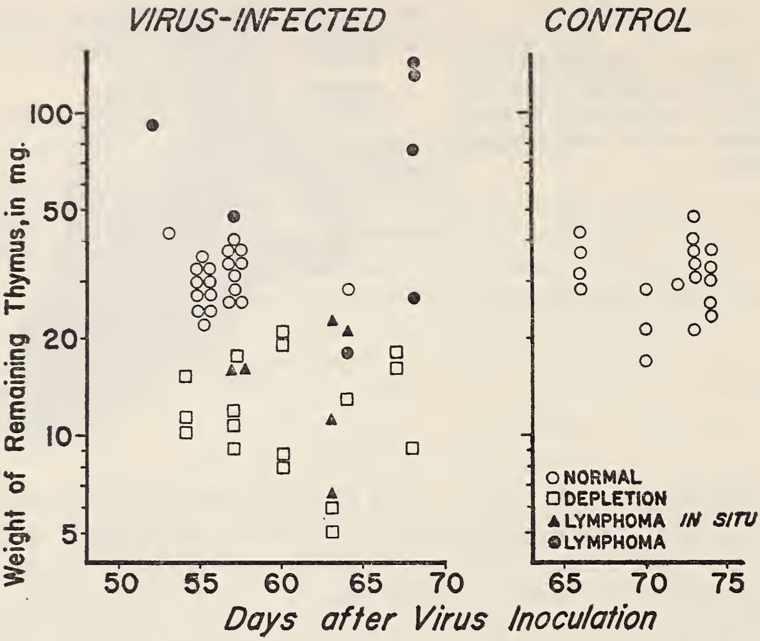
TEXT-FIGURE 2.—Weight and histologic diagnosis of the remaining thymus from unilaterally thymectomized, Rich-virus-inoculated mice and unilaterally thymectomized, uninoculated, control mice.

other morphologists, who noted a preneoplastic lymphocyte depletion or atrophy (*21–25*).

It was of interest to see if the thymic lymphomas induced by radiation had a pathogenesis similar to that of lymphomas induced by viral agents. In one study (*26*), 150 C57BL mice were irradiated by the method of Kaplan (*27*) and killed at intervals over 85 days following the first radiation. At autopsy, the thymuses were separately weighed and serially sectioned for histologic examination. These thymuses were grouped according to histologic appearance into four categories, by criteria previously cited (*15*). Text-figure 4 shows the results obtained when the mean combined (right and left) thymus weight is charted against time. The sharp decrease in mean combined thymic weight and the subsequent compensatory regenerative weight increase immediately following each dose of radiation have been previously reported by Kaplan and Brown (*28*). These wide alternate swings in weight of the thymic tissue during the 4 weeks of radiation were histologically reflected by massive lymphocyte destruction followed by proliferation and differentiation into new small lymphocytes. In text-figure 4, the curve representing the mean combined thymic weight of the irradiated animals between the last radiation (day 21) and the immediate preneoplastic phase (day 42) remains relatively level, ranging between 20 and 32 mg. After day 42, however, the


NORMAL RIGHT THYMUS
LYMPHOCYTE DEPLETION
RETICULUM CELLS
LYMPHOCYTES
CORTEX
MEDULLA
1
2
3
4
LYMPHOMA *IN SITU*
LYMPHOMA

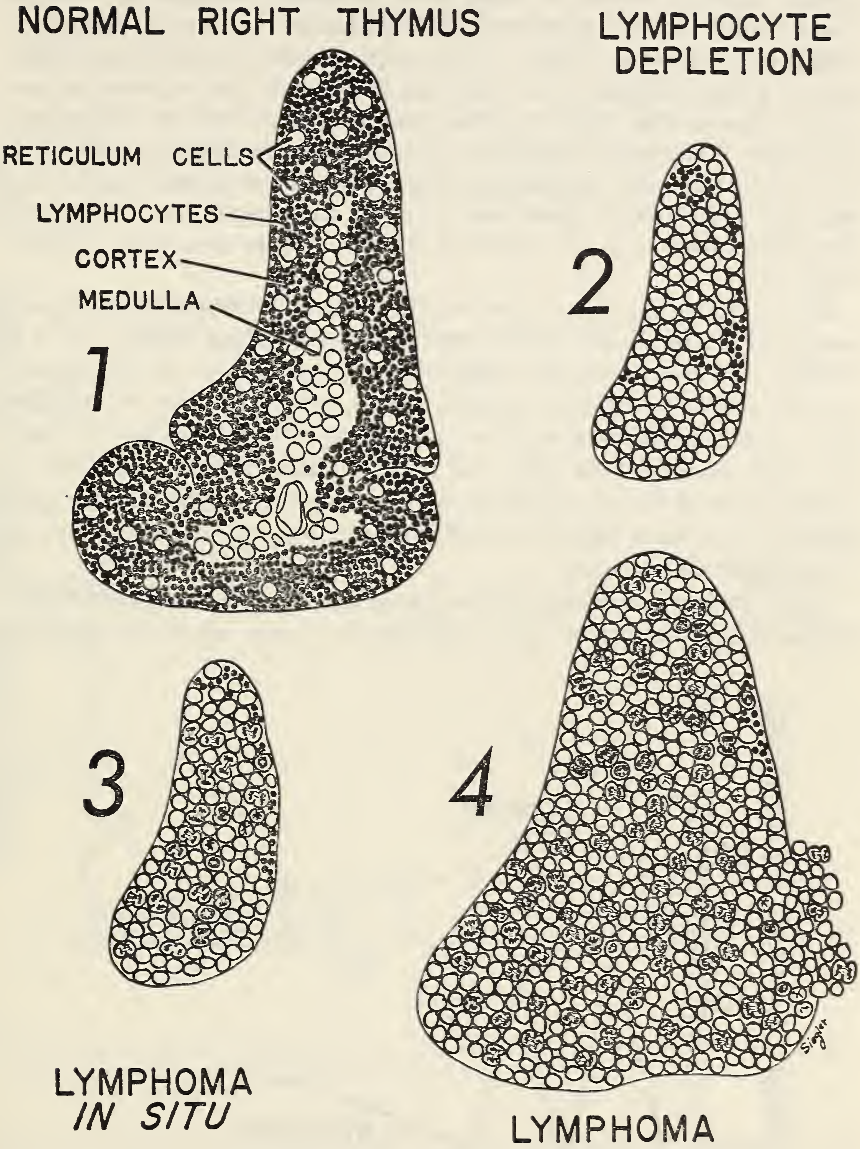
TEXT-FIGURE 3.—Suggested sequence for lymphoma change in the single remaining thymus of unilaterally thymectomized, Rich-virus-inoculated mice. The histologic representation is taken from the stained slides, examples of which are illustrated in figures 6, 7, and 8. The relative size of the remaining thymus is taken from the thymic weights given in text-figure 2.



TEXT-FIGURE 2.—Weight and histologic diagnosis of the remaining thymus from unilaterally thymectomized, Rich-virus-inoculated mice and unilaterally thymectomized, uninoculated, control mice.

other morphologists, who noted a preneoplastic lymphocyte depletion or atrophy (*21–25*).

It was of interest to see if the thymic lymphomas induced by radiation had a pathogenesis similar to that of lymphomas induced by viral agents. In one study (*26*), 150 C57BL mice were irradiated by the method of Kaplan (*27*) and killed at intervals over 85 days following the first radiation. At autopsy, the thymuses were separately weighed and serially sectioned for histologic examination. These thymuses were grouped according to histologic appearance into four categories, by criteria previously cited (*15*). Text-figure 4 shows the results obtained when the mean combined (right and left) thymus weight is charted against time. The sharp decrease in mean combined thymic weight and the subsequent compensatory regenerative weight increase immediately following each dose of radiation have been previously reported by Kaplan and Brown (*28*). These wide alternate swings in weight of the thymic tissue during the 4 weeks of radiation were histologically reflected by massive lymphocyte destruction followed by proliferation and differentiation into new small lymphocytes. In text-figure 4, the curve representing the mean combined thymic weight of the irradiated animals between the last radiation (day 21) and the immediate preneoplastic phase (day 42) remains relatively level, ranging between 20 and 32 mg. After day 42, however, the

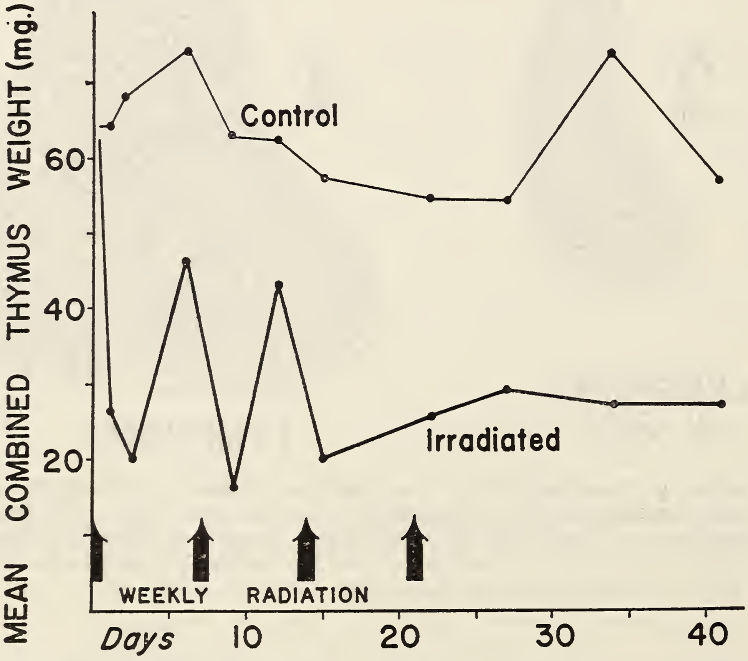

NORMAL RIGHT THYMUS
RETICULUM CELLS
LYMPHOCYTES
CORTEX
MEDULLA
1
LYMPHOCYTE DEPLETION
2
3
LYMPHOMA *IN SITU*
4
LYMPHOMA
Siegler

TEXT-FIGURE 3.—Suggested sequence for lymphoma change in the single remaining thymus of unilaterally thymectomized, Rich-virus-inoculated mice. The histologic representation is taken from the stained slides, examples of which are illustrated in figures 6, 7, and 8. The relative size of the remaining thymus is taken from the thymic weights given in text-figure 2.

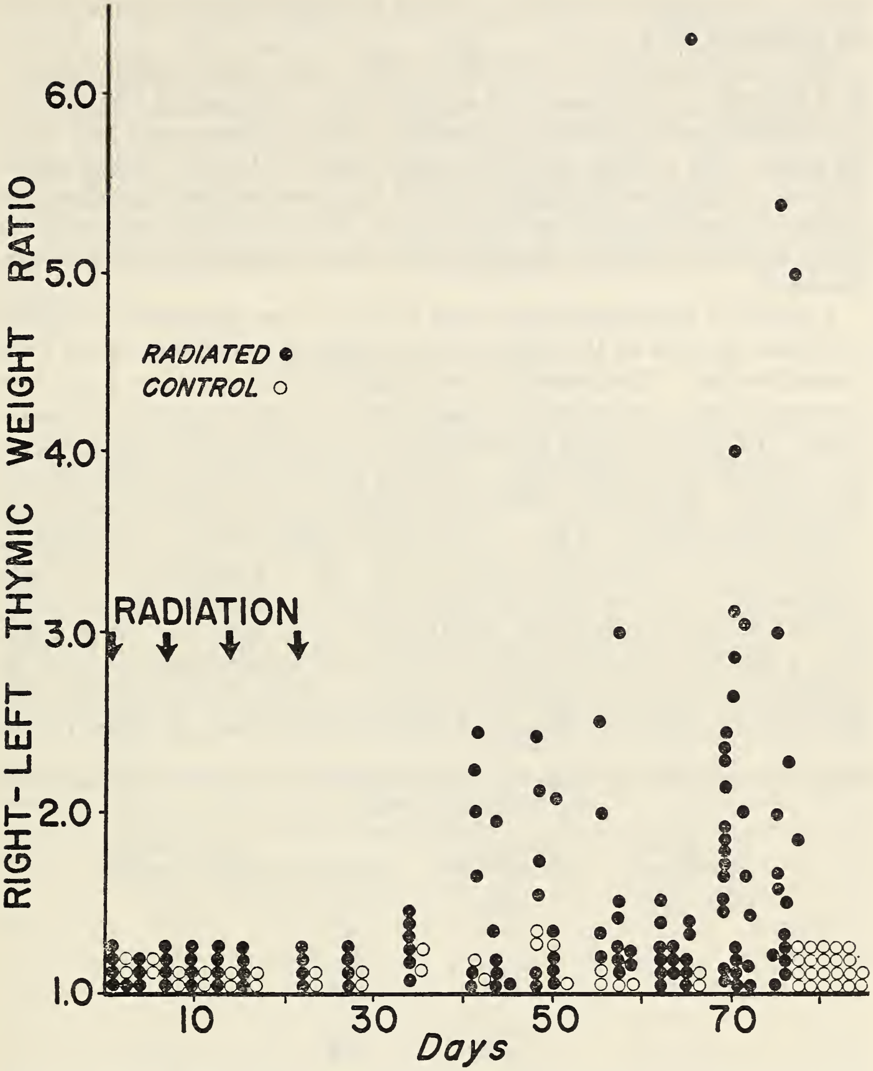
mean combined thymic weight could no longer be used as an index of thymic kinetics, because in many cases unilateral changes had occurred which would not be quantitatively represented by simply adding the weights of the two thymuses. An example of such a preneoplastic specimen is given in figure 9, in which the left thymus has regenerated normally, whereas the right has either remained contracted or undergone a unilateral lymphocytic depletion following postradiation regeneration. One method adopted to quantitatively represent the thymic kinetics during this preneoplastic phase was to chart the *weight ratio* of the two thymuses, rather than the combined weight. These data appear in text-figure 5.

Each point on the chart represents the ratio of the weight of one thymus compared to the weight of the opposite thymus of an animal. It is of interest that the *immediate* effects of radiation, shown in text-figure 4 as wide alternate swings in weight, may be seen in text-figure 5 to affect both thymuses synchronously, as judged by the ratio values of unity. The later *preneoplastic* effects of the radiation, however (text-fig. 5), appear to affect the two thymuses *asynchronously*, as judged by the great number of animals with large differences in weight (and histology) between their two thymuses.

Serial study of the histologic changes in the thymuses of irradiated mice indicated that the tumor arose unilaterally in only one of the thymuses



TEXT-FIGURE 4.—Mean combined thymic weight of irradiated and control mice.



TEXT-FIGURE 5.—Weight ratio of the two thymuses of irradiated animals (*solid circle*) and unirradiated control animals (*open circle*).

(*26*). Further, because the weight of each thymus was recorded, it was possible to demonstrate that the tumor arose in the *small*, *contracted* thymus, rather than in a large, hyperplastic thymus, as reported by others (*29*, *30*). There are, therefore, many similarities in the pathogenesis of the radiation-induced and the virus-induced thymic lymphomas.

Cases of unilateral thymic lymphoma have been reported by Rappaport and Baroni (*22*) in mice given benzanthracene and by Ito *et al.* (*24*) in mice given urethan. It is of interest that this unilateral tumor evolution is not confined to mammals but has been documented to occur in fish by Friedman (*31*).

This puzzling *unilateral* evolution of the tumor is of interest, because it is difficult to understand how a systemically acting agent like a virus, or radiation, or a chemical, can produce a tumor in one organ and spare its mate. The problem posed by these data is: Does the leukemogenic virus act *directly* on the thymic cells of a thymus, or does it produce the tumor by an entirely indirect mechanism? If one is dealing with a direct effect, then it is difficult to see why both thymuses were not simultaneously involved.

A series of experiments have been started in our laboratory, designed to follow the fate of the virus from the time of inoculation to the time tumor develops. The first study was designed to determine how long after intraperitoneal inoculation it would take for virus to appear in thymic tissue. Preliminary data, presented in table 2, indicate that virus is present in the thymuses on day zero, within a few hours after intraperitoneal inoculation. Shortly, we will have information on the relative amount of virus in each thymus, to see if quantitative differences can be detected to account for the unilateral tumors found. If, as Lieberman *et al.* have reported (*32*, *33*), the virus directly affects the individual thymic cells, and if it should be found that each thymus has the same amount of virus, then a possible explanation for the unilateral tumor change may lie in differences between the thymuses themselves. Reasoning by analogy with other paired organs, it may be remembered that the two other paired

TABLE 2.—Assay of virus content of both thymuses after intraperitoneal inoculation of input virus

Days after inoculation	Dilution of thymus tissue	Incidence	Latent period in days (median)
Input virus	10^{-1}	7/7	97
	10^{-3}	5/7	119
0	10^{-1}	7/7	83
	10^{-3}	1/5	—
1	10^{-1}	4/8	90
	10^{-3}	0/8	—
4	10^{-1}	5/7	90
	10^{-3}	1/3	—
6	10^{-1}	6/7	91
	10^{-3}	2/8	—
14	10^{-1}	7/7	71
	10^{-3}	6/6	76

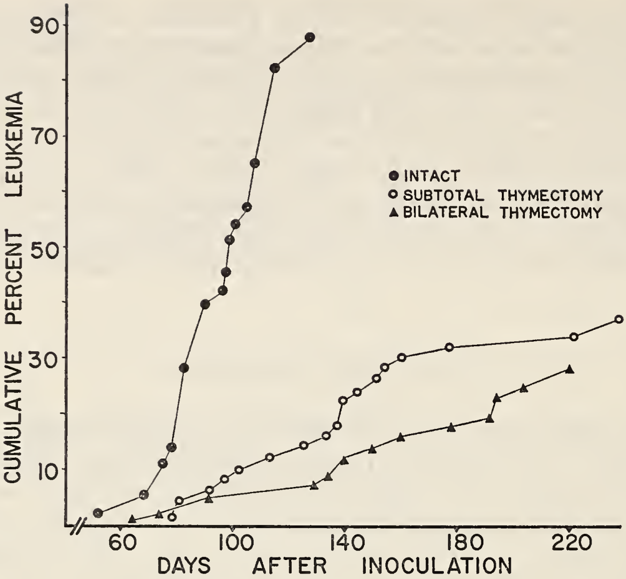
Subsequent to the presentation of this report we have confirmed the results of Lieberman *et al.* (*32, 33*) indicating a *direct* action of the virus on thymic cells. (Studies on the mechanism of action of thymic leukemogenic virus. Siegler, R., and Rich, M. A., Proc. Soc. Exp. Biol. Med. In press.) With regard to the explanation for the unilateral thymic tumors, preliminary data from our laboratory indicate that as early as 3 days after intraperitoneal virus inoculation one thymus, right or left, usually contains significantly more virus than its mate. (Early development of murine leukemia virus in thymus, Masurovsky, E. B., and Rich, M. A., Fed. Proc. 25: 375, 1966.)

organs in the body concerned with the generation of large numbers of cells, namely the testes and the ovaries, pass through their physiologic cycles in an alternate manner. Thus, in higher mammals, only one ovary ovulates at one time, and while quantitative studies are not available, it may be readily verified that in man the testes alternate in size over long periods, and that one is always larger than the other. The possibility, therefore, may exist that, while the two thymuses histologically are indistinguishable in a young animal, some subtle *physiologic* differences exist between them, such that one is more receptive than the other at a given time.

MYELOID LEUKEMIA

The last large group of the leukemias is myeloid leukemia. When the thymic type of leukemogenic viruses is inoculated into thymectomized mice or into older, more resistant intact mice, granulocytic leukemia or reticulum cell sarcoma often occurs (*19, 34*). Yellow-green pigmentation, in varying degrees, in the tumor-infiltrated organs is often present, designated "chloroleukemia." To determine the pathogenesis of these granulocytic leukemias, Rich virus was inoculated into newborn mice thymectomized at 3 to 5 days of age. Two separate studies were completed. The first was an *incidence* study, in which animals were killed only after they had developed obvious tumor (*19*). The second was a *pathogenesis* study (*35*), in which animals were killed at regular intervals *before* clinical tumors were apparent, to study the evolving tumor. Data for the incidence study are given in text-figure 6, which plots the cumulative % of animals developing leukemia against time. Intact, virus-inoculated mice (solid circles) developed *lymphoid* leukemia with a short latency of 60 to 80 days. Thymectomized, virus-inoculated mice, which were found on microscopic examination to contain thymus fragments, were designated "subtotally thymectomized," and are plotted in open circles. These mice developed primarily lymphoid leukemia, although in two instances myeloid tumor was found in the spleen and lymphoid tumor was found in residual thymic fragments and mediastinal nodes. The presence of both the myeloid and lymphoid forms of the neoplasia in virus-inoculated mice has been described by Fiore-Donati and Chieco-Bianchi (*36*). Mice which were on serial section histologically free of thymic fragments were termed "totally thymectomized," and are represented by solid triangles. These mice developed only *myeloid* leukemias. From text-figure 6, it may be observed that the latent period for the myeloid leukemia is longer than for the lymphoid form.

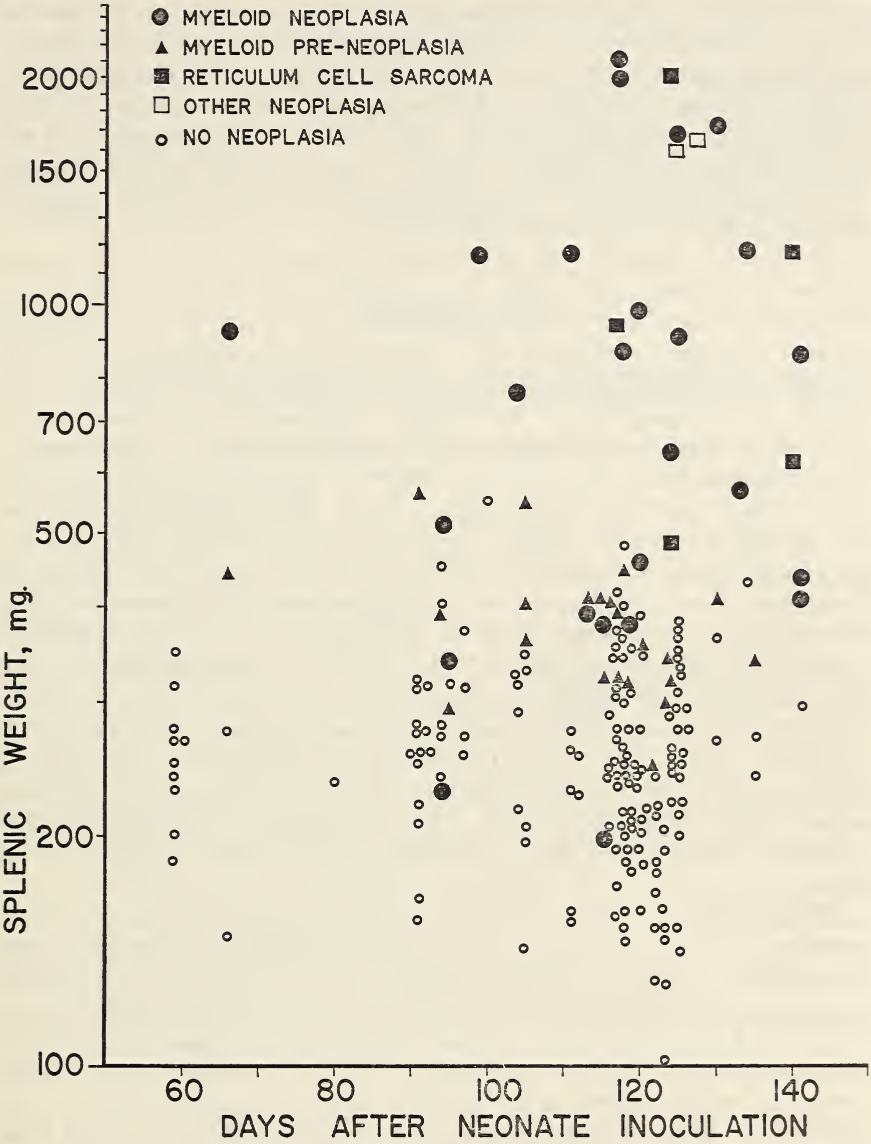
By optical measurement and computation of the mass of remaining thymic tissue in the "subtotally thymectomized" group, it was found that less than 1 mg of residual thymic tissue was sufficient to produce a lymphoma. Since many of these small thymic fragments were discovered only by *serial section* of mediastinal tissue and since lymphoma cells may



TEXT-FIGURE 6.—Cumulative % leukemia in Rich-virus-inoculated mice.

disseminate from a tumor-filled thymus *before* gross enlargement of that thymus occurs (*15*), the existence of nonthymic lymphomas developing in "thymectomized" mice should be accepted with caution unless the presence of microscopic thymic fragments can be eliminated by serial section.

The *pathogenesis* of the evolving myeloid leukemia was studied in 230 totally thymectomized, virus-inoculated mice. The results, reported here briefly, will appear in expanded form in another publication (*35*). These animals were killed between 60 and 140 days after inoculation, the spleen and axillary lymph nodes weighed, and sections prepared of the mediastinal tissues, spleen, sternal marrow, lymph nodes, kidneys, and liver. On the basis of histologic findings and organ weight, three groups were defined. In the first group, 162 of the 217 thymectomized, virus-inoculated mice showed no specific histologic alterations and were regarded as *tumor free*. Of the remaining 55 animals, 25 had *overt myeloid leukemia*, as judged by 1) marked enlargement of the spleen and lymph nodes, 2) histologic replacement of the spleen and other organs by myeloid tumor tissue, and 3) myeloblasts in the peripheral bloodstream in large numbers. An additional 22 mice had slight gross enlargement of the spleen and histologically showed extensive proliferation of myeloblasts in the spleen red pulp. In some instances the sternal marrow of these animals showed an increase in myeloblasts, while in other cases it did not. The changes in this group of 22 mice were designated *preneoplastic myeloid* changes. Five cases of reticulum cell sarcoma and 3 cases of undetermined monocytic tumors of the spleen were also found. The weight of the spleen and histologic diagnosis of these 217 animals are presented in text-figure 7.



TEXT-FIGURE 7.—Histologic diagnosis and splenic weight of thymectomized, Rich-virus-inoculated mice on day of sacrifice.

In figure 13, the normal histology of the spleen is illustrated for purposes of comparison. The splenic tissue may be divided on a histologic basis into the lymphoid follicles (arrows) and the red pulp. Figure 14 shows a spleen from a thymectomized, virus-inoculated mouse, in which there is an increase in the number of primitive myeloblasts of the red pulp. Figure 15 illustrates a spleen in which more extensive proliferation of myeloblasts has occurred in the red pulp, resulting in an increase in splenic weight. These changes were termed "myeloid preneoplasia." The lymphoid follicles did not take part in the proliferation of cells of the red pulp. With continued proliferation of these myeloblasts, the spleens enlarged greatly, often weighing 1 to 2 g. Early overt myeloid neoplasia is exemplified by the spleen (fig. 16), where its entire red pulp has been replaced by confluent proliferation of myeloblasts. A few small splenic lymphoid follicles persisted but showed no proliferation.

It was of interest to note the striking appearance of the lymph nodes in the preleukemic mice (figs. 10, 11, and 12). The nodes, slightly enlarged, but free of tumor, show under low power prominent and enlarged central medullary cords (fig. 10). Under higher magnification (fig. 11), it may be seen that these medullary cords, instead of containing their usual sheath of lymphocytes, now are packed with neutrophils. These neutrophils do not seem to be associated with the other changes normally seen in acute inflammation, and there is no obvious explanation for their presence in such large numbers in the medullary cords. It may be remembered that the medullary cords of lymph nodes contain a centrally located, postcapillary venule, and many neutrophils are to be seen in the lumina of these specialized venules. It was our impression that these neutrophils were selectively taken up by the postcapillary venules of the medullary cords in the preleukemic period. In the lower portion of figure 12 can be seen a central medullary cord filled with mature lymphocytes; the cortex of that node has already been largely replaced by tumor myeloblasts (top of figure). Data from this study are still being analyzed, but preliminary observations suggest that a definite fall in the peripheral white count occurs, which is associated with the accumulation of neutrophils in the medullary cords before the myeloid tumor cells appear in the peripheral blood.

With further proliferation of the splenic myeloid cells (fig. 17), large numbers of these primitive cells detached and entered the venous sinuses, eventually to appear in the portal vein of the liver (fig. 18) in a manner similar to that illustrated for the Friend and Rauscher leukemias (figs. 2 and 3). At the same time that these myeloblasts of splenic origin were accumulating in the liver, scattered foci of similar tumor cells were observed in lymph nodes in a few animals. Except in extreme cases, it was difficult to state whether individual myeloblasts observed in the bone marrow were those normally found there or represented neoplastic cells. In general, the marrow changes lagged behind the splenic changes. In terms of absolute size, the contribution of tumor cells from the spleen probably outnumbered that from the marrow. When tumor was far advanced in the spleen, the marrow was almost always involved, as illustrated by the specimen of sternum in figure 19. The proliferation of tumor cells outside the medullary spaces, as seen here, caused elevation of the periosteum and exosteal new bone formation (arrow), a finding so characteristic of human myeloid leukemia. It was of interest to note that myelofibrosis, often seen in association with human myeloid leukemias, occurred very frequently in far advanced cases (fig. 20).

In summary, it may be stated that inoculation of mice with suitable viral agents reproduces the entire range of morphologic varieties of the disease leukemia seen in man. Study of the pathogenesis of this disease in mice may be expected to contribute toward our over-all understanding of its biologic essence and the eventual eradication of its counterpart in man.

REFERENCES

(*1*) McEndy, D. P., Boon, M. E., and Furth, J.: On the role of thymus, spleen, and gonads in the development of leukemia in a high-leukemia stock of mice. Cancer Res 4: 377–383, 1944.

(*2*) Dunn, T. B.: Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J Nat Cancer Inst 14: 1281–1433, 1954.

(*3*) Virchow, R.: Weisses blut. Froriep's Notizen. Nr. 780, 33: 151, 1845.

(*4*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*5*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307–318, 1956.

(*6*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*7*) Metcalf, D., Furth, J., and Buffett, R. F.: Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res 19: 52–58, 1959.

(*8*) Siegler, R., and Rich, M. A.: Comparative pathogenesis of murine viral lymphoma. Cancer Res 24: 1406–1417, 1964.

(*9*) Mirand, E. A.: Erythropoietic response of animals infected with various strains of Friend virus. Nat Cancer Inst Monogr 22: 483–503, 1966.

(*10*) Buffett, R. F., and Furth, J.: A transplantable reticulum-cell sarcoma variant of Friend's viral leukemia. Cancer Res 19: 1063–1069, 1959.

(*11*) di Guglielmo, G.: Les maladies érythrémiques. Rev Hématol 1: 355, 1946.

(*12*) Chamorro, A.: Origine et transmission de la leucémie de Friend. Bull Assoc Franc Cancer 49: 399–415, 1962.

(*13*) Gross, L.: Pathogenic properties, and "vertical" transmission of the mouse leukemia agent. Proc Soc Exp Biol Med 78: 342–348, 1951.

(*14*) Siegler, R., and Rich, M. A.: Unilateral histogenesis of AKR thymic lymphoma. Cancer Res 23: 1669–1678, 1963.

(*15*) Siegler, R., Geldner, J., and Rich, M. A.: Histogenesis of thymic lymphoma induced by a murine leukemia virus (Rich). Cancer Res 24: 444–459, 1964.

(*16*) Siegler, R.: The morphology of the thymuses and their relation to leukemia. *In* The Thymus in Immunobiology (Good, R. A., and Gabrielsen, A. E., eds.). New York, Harper & Row, 1964, p 623.

(*17*) Hassall, A. H.: The Microscopic Anatomy of the Human Body in Health and Disease. New York, Samuel S. & William Wood Publishers, vol 1, 1855, p 484.

(*18*) Appleton, A. B.: Ductless glands. *In* Cunningham's Text-Book of Anatomy, 9th ed. (Brash, J. C., ed.). London, Oxford Univ Press, 1951, pp 820–821.

(*19*) Siegler, R., and Rich, M. A.: Influence of thymic mass on murine viral leukaemogenesis. Nature (London) 209: 313–314, 1966.

(*20*) Rich, M. A., Geldner, J., and Meyers, P.: Studies on murine leukemia. J Nat Cancer Inst 35: 523–536, 1965.

(*21*) Dunn, T. B., Moloney, J. B., Green, A. W. and Arnold, B.: Pathogenesis of a virus-induced leukemia in mice. J Nat Cancer Inst 26: 189–221, 1961.

(*22*) Rappaport, H., and Baroni, C.: A study of the pathogenesis of malignant lymphoma induced in the Swiss mouse by 7,12-dimethylbenz[*a*]anthracene injected at birth. Cancer Res 22: 1067–1074, 1962.

(*23*) Goodman, S. B., and Block, M. H.: The histogenesis of Gross's viral induced mouse leukemia. Cancer Res 23: 1634–1640, 1963.

(*24*) Ito, T., Hoshino, T., and Sawauchi, K.: Pathogenesis of thymic lymphoma induced by urethan in mice. Z Krebsforsch 66: 267–273, 1964.

(*25*) Arnesen, K.: Preleukemic and early leukemic changes in the thymus of mice. Acta Path Microbiol Scand 43: 350–364, 1958.

(*26*) Siegler, R., and Rich, M. A.: Pathogenesis of murine radiation lymphoma. Proc Amer Assoc Cancer Res 6: 59, 1965.

(*27*) Kaplan, H. S., Carnes, W. H., Brown, M. B., and Hirsch, B. B.: Indirect induction of lymphomas in irradiated mice; tumor incidence and morphology in mice bearing nonirradiated thymic grafts. Cancer Res 16: 422–425, 1956.

(*28*) Kaplan, H. S., and Brown, M. B.: Radiation injury and regeneration in lymphoid tissues. *In* The Leukemias; Etiology, Pathophysiology, and Treatment (Rebuck, J., Bethell, F., and Monto, R., eds.). New York, Academic Press, Inc., 1957, chapt 9.

(*29*) Carnes, W., and Kaplan, H. S.: Histogenesis of lymphomas in irradiated C57BL mice bearing non-irradiated thymic grafts. Proc Amer Assoc Cancer Res 2: 99, 1956.

(*30*) Kaplan, H. S.: Early microscopic diagnosis of lymphosarcoma *in situ* in thymus of irradiated mice. Fed Proc 19: 399, 1960.

(*31*) Friedman, L. R.: A study of normal and malignant thymus tissue of the teleost *Astyanax mexicanus* in tissue culture. Bull Amer Mus Nat Hist 124: 69–100, 1962.

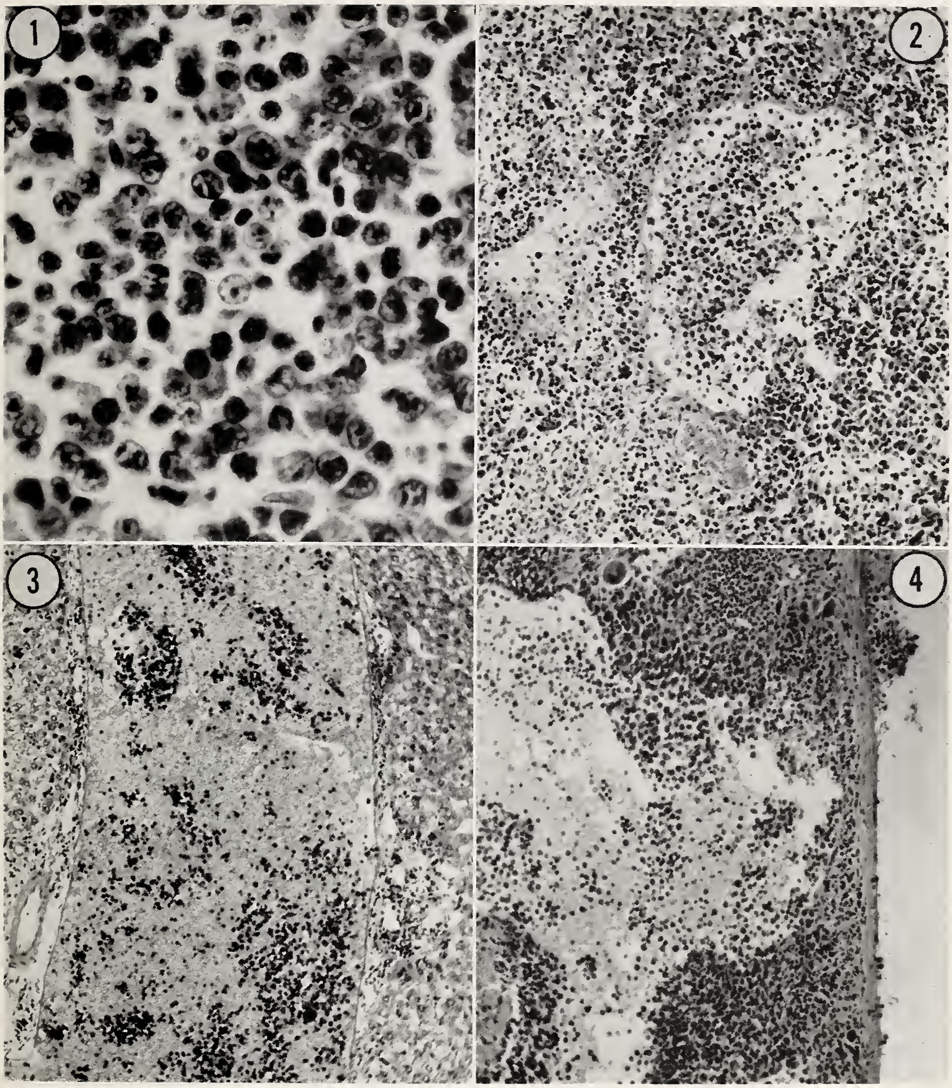
(*32*) Lieberman, M., Haran-Ghera, N., and Kaplan, H. S.: Direct action of a leukemogenic virus on the thymus. Proc Amer Assoc Cancer Res 6: 40, 1965.

(*33*) Lieberman, M., and Kaplan, H. S.: Lymphoid tumor induction by mouse thymocytes infected *in vitro* with radiation leukemia virus. Nat Cancer Inst Monogr 22: 549–557, 1966.

(*34*) Gross, L.: Development of myeloid (chloro-) leukemia in thymectomized C3H mice following inoculation of lymphatic leukemia virus. Proc Soc Exp Biol Med 103: 509–514, 1960.

(*35*) Siegler, R., and Rich, M. A.: Pathogenesis of viral-induced murine myeloid leukemia. Fed Proc 25: 477, 1966.

(*36*) Fiore-Donati, L., and Chieco-Bianchi, L.: Influence of host factors on development and type of leukemia induced in mice by Graffi virus. J Nat Cancer Inst 32: 1083–1107, 1964.



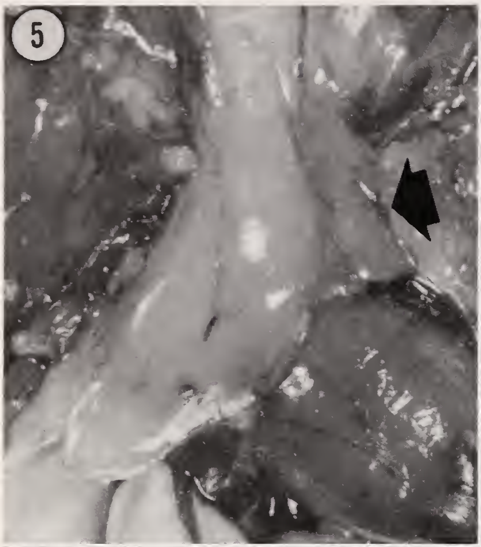
ALL STAINING IS WITH HEMATOXYLIN AND EOSIN.

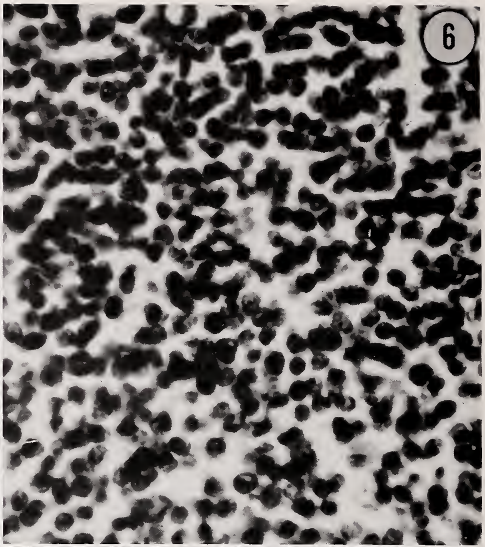
Figure 1.—High-power view of red pulp of spleen of mouse inoculated 30 days previously with Friend virus. The proliferating cells are primitive cells of the erythrocyte series. × 500

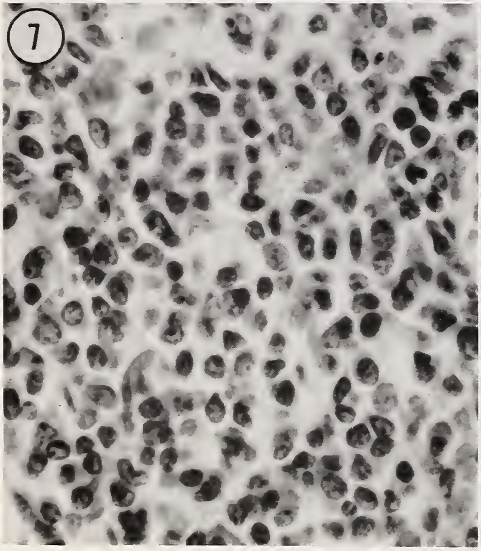
Figure 2.—Orientation view of Friend virus-infected spleen, to show primitive erythroblasts entering branch of splenic venous sinus. From Siegler and Rich (*8*). × 150

Figure 3.—Liver of animal shown in figure 2. Splenic erythroblasts in intrahepatic branch of portal vein. From Siegler and Rich (*8*). × 100

Figure 4.—Spleen of animal infected with Rauscher virus 50 days previously. Evacuation of masses of splenic erythroblasts plus hemostatic pressure created large blood lake. *Note* close relation of hemorrhage to capsule at *right*. Rupture of spleen by this mechanism frequently caused death. × 100







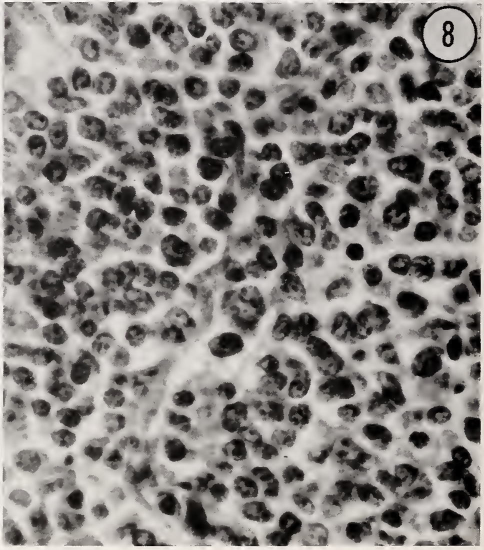
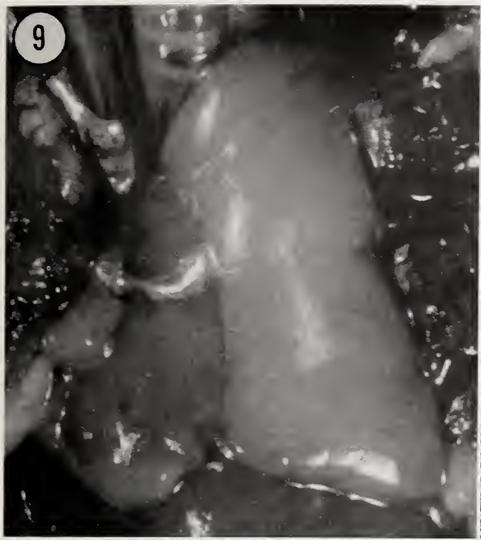


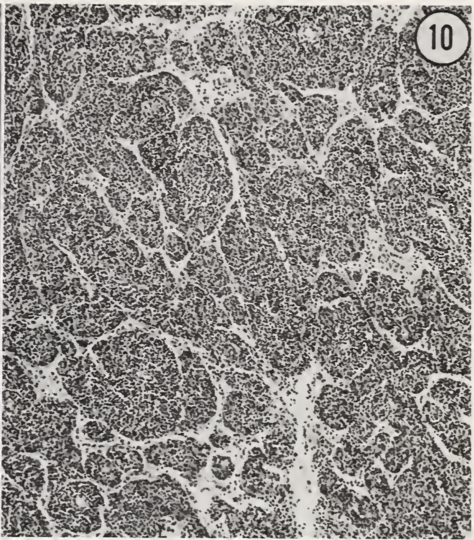
FIGURE 5.—Unilateral lymphocyte depletion. The thymuses of an 8-month-old AKR mouse. The right thymus (*left*) weighs 57 mg, which is normal for the age, sex, and strain. The left thymus (*arrow*) weighs 12 mg, and histologically had lost all its lymphocytes. From Siegler and Rich (*14*). × 10

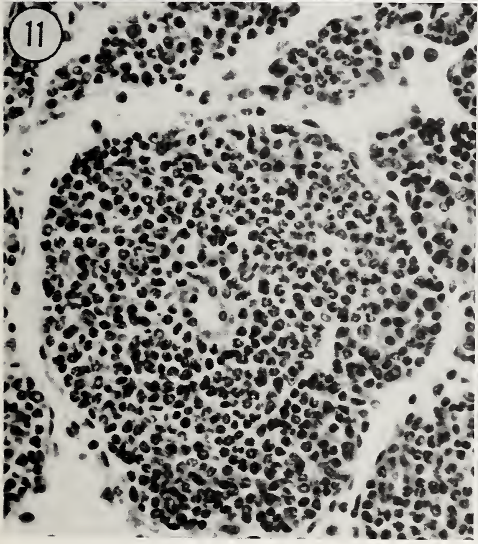
FIGURE 6.—Cortex of control remaining 39 mg thymus from unilaterally thymectomized Swiss ICR/Ha mouse. × 400

FIGURE 7.—Cortex of Rich-virus-infected remaining 6 mg thymus 40 days after inoculation. *Note* loss of lymphocytes and relative increase in number of matrix reticulum epithelial cells due to contracture. × 400

FIGURE 8.—Cortical region of 17 mg remaining thymus 65 days after unilateral thymectomy and Rich virus inoculation. This tumor-replaced thymic mass is one half the size of the control thymus shown in figure 6. × 400







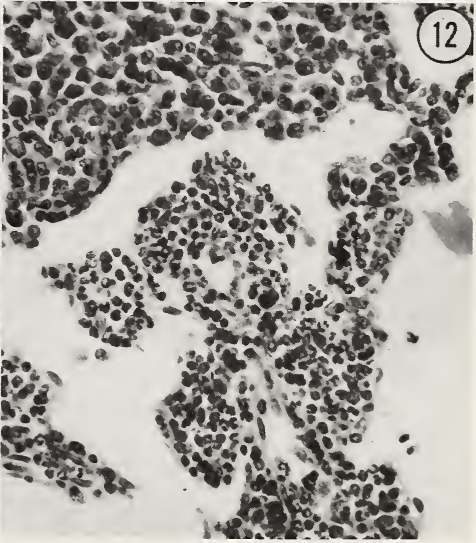


Figure 9.—Preneoplastic changes in the thymuses of C57BL mouse 40 days after last radiation. The large, 63 mg, left thymus has regenerated normally, whereas the small, 10 mg, right thymus has remained contracted. Histologically, cells morphologically similar to lymphoma cells are present in the small, contracted right thymus, whereas the normal, regenerated left thymus contains only small lymphocytes. × 10

Figure 10.—Medulla of lymph node of Rich-virus-inoculated, totally thymectomized Swiss ICR/Ha mouse, showing prominent and enlarged medullary cords. × 80

Figure 11.—Single medullary cord from figure 10, illustrating large numbers of mature neutrophils and absence of usual signs of acute inflammation. × 200

Figure 12.—Node from mouse with Rich-virus-induced myeloid leukemia, showing myeloblasts replacing cortical region (*above*), while mature neutrophils persist in medullary cord (*below*). × 200

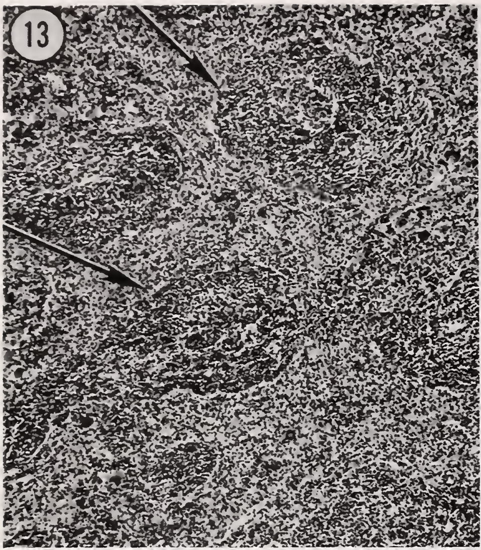
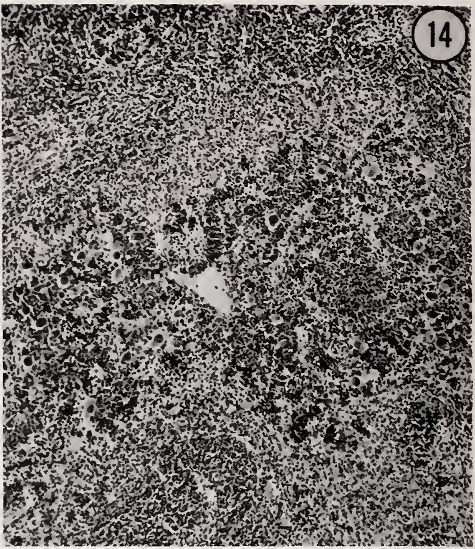
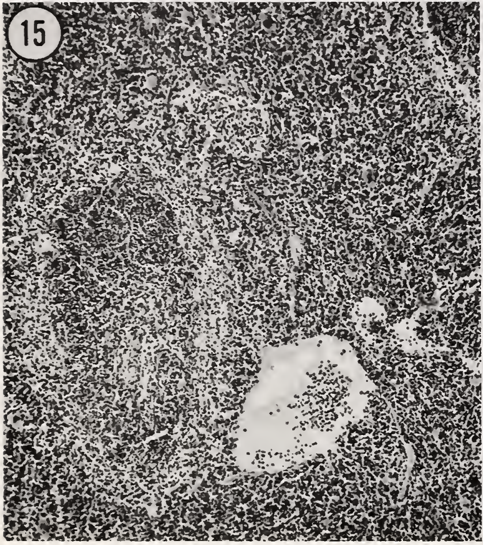
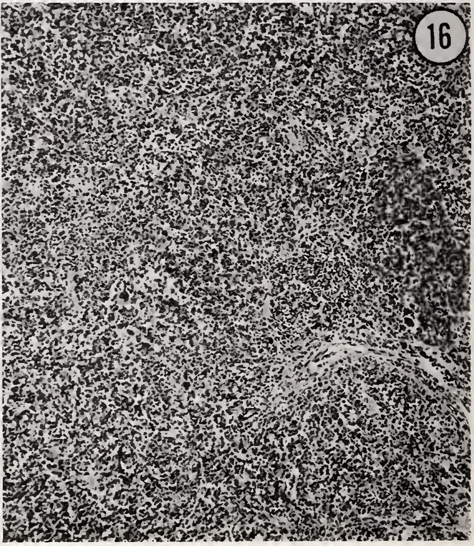
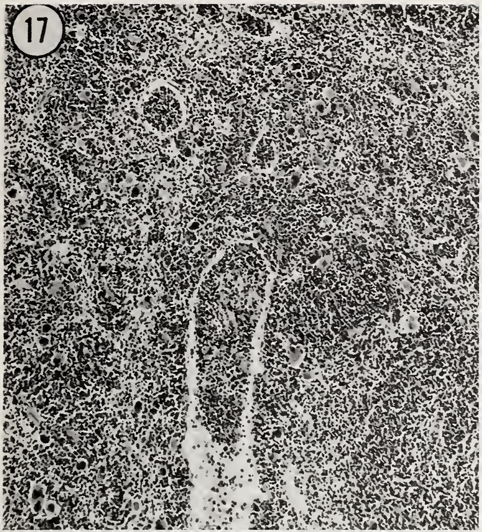





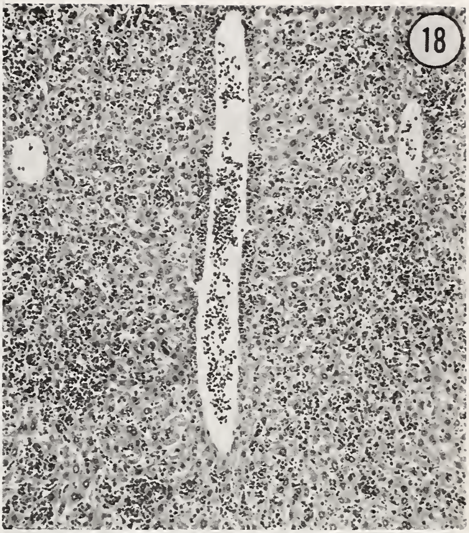
FIGURE 13.—Survey view of control spleen, illustrating normal relationship of follicles (*arrows*) to surrounding red pulp. Thymectomized control mouse. × 50

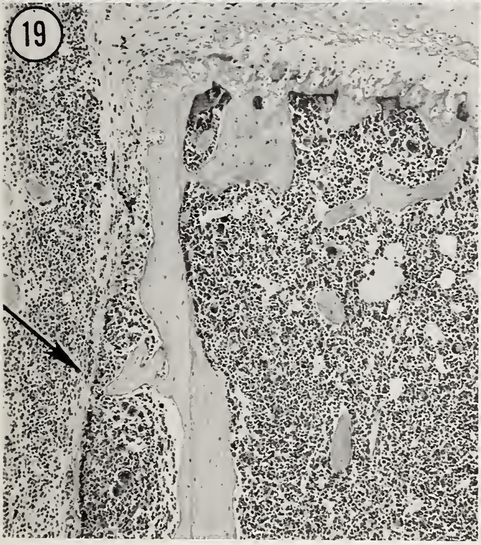
FIGURE 14.—Rich-virus-infected, thymectomized mouse spleen, illustrating early increase in volume of red pulp and slight dilatation of branch of splenic venous sinus. × 50

FIGURE 15.—Myeloid preneoplasia. Continued myeloblast proliferation in red pulp, with early detachment of primitive cells to enter the now enlarged venous sinus (*lower right*). The lymphoid follicle at *left* shows no proliferative changes under high power. Spleens of this appearance weighed between 400 and 700 mg. × 50

FIGURE 16.—Overt myeloid neoplasia. Complete replacement of splenic tissue by primitive myeloid cells, associated with very early peripheralization of myeloblasts ("leukemia"). Spleens of this appearance weighed between 700 and 1500 mg. × 50


17


18


19

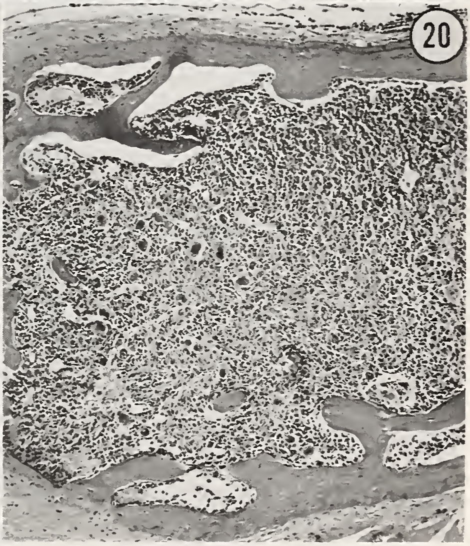

20

Figure 17.—Massive splenomegaly in advanced myeloid neoplasia with leukemia. Large numbers of primitive myeloid cells have detached from the splenic red pulp and may be seen in the splenic venous sinuses. The liver of this animal is shown in figure 8. × 50

Figure 18.—Liver from animal shown in figure 17. Primitive myeloblasts of splenic origin lie in the interhepatic branches of the portal vein. Other myeloblasts are present in the liver sinusoids, through which they pass to enter the peripheral circulation to constitute the "leukemia."

Figure 19.—Extensive replacement of sternal bone marrow by neoplastic myeloblasts (*right*). The periosteum (*arrow*) is elevated by extramedullary tumor, which has produced exosteal new bone formation. × 50

Figure 20.—Replacement of tumor-filled medullary cavity by fibrous tissue (myelofibrosis). × 50

DISCUSSION

Dr. Dunn: I want to congratulate Dr. Siegler on the clearness with which he observes. It is certainly a great satisfaction to find that something has been considered histologically in this whole concern with tumor viruses in mice. Once we agree on what we see, the interpretation becomes relatively unimportant.

I certainly agree with Dr. Siegler's observations on the variation in the response of what we have previously called two lobes of the thymus to these various agents. Although we might also compare the effects of inanition, and of cortisone and various other substances, I have in mind particularly the estrogenic hormones. I suspect (although I've never tried) there are many circumstances under which the two lobes or two thymuses would react identically.

Dr. Kirsten: Dr. Siegler, in the thymectomized or partially thymectomized mouse, previously injected with virus, did I understand correctly that there are no discernible changes in the bone marrow when splenic myeloid proliferation has already begun?

Dr. Siegler: No, I said that in the myeloid leukemias we did not find obvious tumor in the bone marrow until tumor was already present in the spleen. There are changes in the bone marrow that I didn't show here. We see an accumulation of neutrophils and an accentuation of primitive elements in the marrow. I emphasized that it is very difficult in these cases of early tumor to say whether the myeloblasts you see in the marrow represent tumor or normally occurring myeloblasts.

Dr. Block: Should animals with large numbers of immature granulocytes growing in an abnormal manner in the spleen be classified as leukemic rather than preleukemic? You are really describing a malignancy in hematopoietic tissues which has not yet spilled into the blood. Abnormalities in the blood are not the disease but merely a reflection of the disease that is already present in the hematopoietic tissues.

On that basis I would think that what you had called preleukemic was already leukemia, merely that it hadn't spilled into the blood, and that the real preleukemic changes are even earlier.

Dr. Siegler: Thank you, Dr. Block, for calling attention to this confusing area. In our presentation we restricted the word "leukemia" to mean the presence of neoplastic hematopoietic cells in the *peripheral blood*. This usage is in accord with that of Virchow (Froriep's Ntz., 33: 151, 1845). The term "preleukemic," then, would refer to any changes, both preneoplastic as well as neoplastic, which occur before *peripheralization* of the neoplastic cells occurs. When you say we have a "leukemia which had not yet spilled into the blood" you phrase the same paradox found in the term "aleukemic leukemia." Even aside from the problem of definition, there is a conceptual problem involved. The histological changes are part of an on-going, continuous process of change. While we can identify with certainty the normal cells at one end and the tumor cells at the other end, the area in between—since it is in transition—is difficult to characterize. It may be preferable merely to regard the intermediate changes as transitory and, in the broadest sense, "preleukemic."

Chairman Furth: What we really need is a technique to identify transformed cells from normal cells.

Dr. Fiore-Donati: When you made unilateral thymectomy did you get myeloid leukemias also?

Dr. Siegler: No. In the unilaterally thymectomized mice the incidence and the latency of the lymphoid leukemia were identical to that of intact mice.

Dr. Upton: First I should like to compliment Dr. Siegler and Dr. Rich for their clear and detailed presentation. Then I should like to inquire about what I understood them to report as a virus-induced atrophy of the thymus. Could you say a little more about how soon this is evident and what you think the mechanism might be?

Dr. Siegler: In viral-induced lymphoma there is a marked thymic lymphocyte depletion in the immediate preneoplastic period. This is usually obvious in only one of the two thymuses, and may not be noticed unless the two thymuses are separately weighed and examined. This preneoplastic thymic lymphocyte depletion is probably part of the physiologic response to the virus. This unilateral thymic depletion may be distinguished from the bilateral thymic lymphocyte depletion produced by the early direct effect of radiation, as I illustrated in the talk, or by any other systemically acting agent, like cortisone or estrogen.

Dr. Metcalf: Just a further word about this preleukemic atrophy of the thymus. It is characteristic of lymphoid leukemia development in AKR and C58 mice and in the chemical-carcinogen-induced and the viral-induced lymphoid leukemias. Recently, we did some interesting audioradiographic studies on the kinetics of cellular proliferation in the thymus of preleukemic mice. The primitive large and medium lymphoid cells divided as rapidly as in the normal thymus, but the small lymphocyte had an abnormally short lifespan. It is for this reason you get the selective depletion of the small lymphocyte population in the preleukemic thymus (Metcalf, D., unpublished data).

Lymphoid Tumor Induction by Mouse Thymocytes Infected *In Vitro* With Radiation Leukemia Virus [1, 2]

MIRIAM LIEBERMAN *and* HENRY S. KAPLAN,
Department of Radiology, Stanford University School of Medicine, Palo Alto, California

STUDIES of the role of the thymus in murine lymphocytic leukemia have amply demonstrated its essential function as the target organ for the disease process, whether induced by radiation, chemical carcinogens, or viruses (*1*, *2*). The present study was undertaken to elucidate further the interaction between thymic cells and the radiation leukemia virus (RLV), a leukemogenic virus extracted from radiogenic lymphomas of strain C57BL mice (*3*, *4*). By injection of lymphoid cells from donor mice of one parental strain, after RLV infection *in vitro*, into thymus grafts from donors of the other parental strain growing in thymectomized, irradiated F_1 hybrid hosts, it was possible to establish that thymic lymphoid cells are the targets for neoplastic transformation by RLV and to assess the influence of several parameters on tumor incidence and transplantation behavior.

MATERIALS AND METHODS

Mice. The graft-bearing hosts, were reciprocal F_1 hybrid crosses between inbred strains C57BL/Ka and BALB/cKa or C3H. Thymus grafts and cell suspensions were provided by animals of the two parental strains.

X irradiation was performed at 250 kv, 15 ma, 0.25 mm Cu + 1.0 mm Al added filter, with a dose rate of 83 r per minute. Mice were given 600 r whole-body irradiation, in 3 weekly doses of 200 r; they were 6 weeks old at the time of first exposure, which took place 1 week after total thymectomy.

Thymus grafts were implanted under the renal capsule promptly after the last X-ray exposure, as previously described (*5*).

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by Public Health Service research grant CA–03352 from the National Cancer Institute.

RLV-infected cell suspensions were provided as follows: 1) Thymocyte suspensions were prepared from day-old donors, or as noted in individual experiments. Thymus glands were removed and pooled in a cell-suspending medium (CSM), which consisted of chilled phosphate-buffered saline (PBS) and 5% fetal bovine serum. They were pushed through a stainless-steel mesh and allowed to stand for a few minutes to let clumps settle. The cell suspension was mixed with an equal volume of RLV preparation and incubated, with occasional stirring, for about 1 hour at room temperature, then placed for an equal time on ice. For removal of unadsorbed virus, the infected cells were washed 3 times with CSM, which reduced the free virus concentration by a factor of about 10^6. The washed cells were resuspended in CSM and counted with the aid of trypan blue; no more than 10% took up the dye. The cell suspension was adjusted so that 0.02 ml contained 4 to 8 × 10^6 cells, and this volume was injected directly into a thymus graft which had been implanted intrarenally 7 to 10 days earlier. 2) Cells from peritoneal exudate were used as controls for the specificity of action of thymic cells. They were obtained by injection of 2 ml of sterile thioglycollate medium intraperitoneally into 2-month-old C57BL mice (*6*). The following day, the peritoneal cavity was washed out with PBS and heparin (5 units/ml). The cells consisted mainly of macrophages, lymphocytes, and polymorphonuclear cells; they were washed once with PBS, resuspended in CSM, and thereafter the procedure was similar to that used for thymocytes.

RLV origin and preparation have been described in previous publications (*3, 4*). Where it was used as a control for the inoculation of infected cells, 0.02 ml of the same preparation of RLV was injected directly into thymic grafts.

RESULTS

Lymphoma Induction by RLV-Infected Cells

Single thymic lobes from newborn BALB/c donors were grafted under the left kidney capsule of thymectomized, irradiated F_1 hybrid mice. The regenerated graft was then inoculated with 1) a preparation of RLV; 2) RLV-infected thymocytes from newborn C57BL mice; or 3) RLV-infected peritoneal cells from adult C57BL donors. Whenever possible, the ensuing lymphoid tumors were transplanted to mice of both parental strains and to F_1 hybrids to assess their genetic origin.

There was little difference in the frequency of tumors in the three treatment groups (78–84%), or in the average latent period (94–110 days), which suggests that infected cells are no less efficient than free virus in producing lymphomas (table 1). From their transplantation behavior, it appears that the tumors induced by free RLV originated predominantly in the thymus graft; the few of host genotype were presumably derived from cells which had invaded the graft (*7, 8*). The tumors resulting from

TABLE 1.—Lymphomas in thymectomized, irradiated (BALB/c × C57BL)F_1 hybrid mice bearing intrarenal BALB/c thymus grafts inoculated with RLV-infected C57BL thymocytes or peritoneal cells

Infected cells	Number of lymphomas/ No. of mice	Percent	Lymphoma genotype/ No. of lymphomas tested: C57BL	BALB/c	F_1
Thymocytes	36/43	84	15/35	12/35	5/35
Peritoneal cells	14/17	82	2/13	6/13	3/13
None (free RLV)	14/18	78	0/11	6/11	3/11

injection of RLV-infected peritoneal cells behaved similarly, except in two instances, in which it is presumed that C57BL lymphocytes in the peritoneal exudate may have taken up the virus and undergone neoplastic transformation. However, when thymus cells infected with RLV were inoculated, tumors of cell-donor origin predominated; those of graft-donor genotype were also numerous, but relatively few were of the host type. It thus appears that cells from peritoneal exudate do adsorb the virus but usually release it again in the thymus graft, making it available to the lymphoid cells there. Thymic lymphocytes not only take up the virus *in vitro*, but, after entering the environment of the thymic graft, may also serve as the target cells for the action of the virus and undergo neoplastic transformation. The gross and microscopic characteristics of the neoplasm were similar, regardless of the means of induction: The thymic graft and other lymphoid organs were extensively invaded by tumor and other tissues were also frequently infiltrated.

The importance of viable thymic epithelium is suggested by several experiments. Thymic cells taken from weanling (*ca.* 30-day-old) mice, rather than from infant donors, have produced no observable difference in tumor yield to date. By contrast, the age of the thymus graft donor seems to be of greater importance. Thymic tissue taken from weanling or adult mice grows poorly, if at all, in the kidney, and virtually no lymphomas developed when infected neonatal thymocytes were inoculated into such grafts. Essentially no tumors were obtained when the graft was omitted and infected thymocytes were injected directly into the kidney, or when the thymus was irradiated, either in the newborn donor or *in vitro*, with a single X-ray dose of 3000 r, immediately before implantation.

Role of Thymic and Host Factors in Leukemogenesis

Thymectomized irradiated F_1 hybrids received a thymus graft from one parental strain, followed by intragraft inoculation of RLV-infected thymic lymphocytes from the other parent. Two parallel experiments were set up: in one, the parent strains were C57BL and BALB/c, both sensitive to the leukemogenic action of RLV (C57BL > BALB/c), in the other, C57BL and C3H, the latter resistant to RLV.

TABLE 2.—Lymphomas in thymectomized, irradiated F_1 hybrid mice bearing intrarenal thymus grafts inoculated with RLV-infected thymocytes from either parental strain

Group	Host genotype	Graft-donor strain	Thymocyte-donor strain	Number of lymphomas/ No. of mice	Percent	Average latent period (days)	Lymphoma Genotype		
							C57BL	BALB/c	F_1
1	(C57BL × BALB/c)F_1	BALB/c	C57BL	20/25	80	115	5/18	4/18	8/18
2	(C57BL × BALB/c)F_1	BALB/c	BALB/c	19/26	73	144	0/17	12/17	4/17
3	(C57BL × BALB/c)F_1	C57BL	BALB/c	24/27	89	100	2/23	18/23	3/23
4	(C57BL × BALB/c)F_1	C57BL	C57BL	23/26	89	100	15/21	0/21	5/21
							C57BL	C3H	F_1
5	(C57BL × C3H)F_1	C3H	C57BL	6/20	30	172	4/6	0/6	0/6
6	(C57BL × C3H)F_1	C3H	C3H	1/18	6	286	ND*	ND	ND
7	(C57BL × C3H)F_1	C57BL	C3H	2/20	10	142	2/2	0/2	0/2
8	(C57BL × C3H)F_1	C57BL	C57BL	6/18	33	109	2/3	0/3	1/3
9	(C57BL × BALB/c)F_1	C57BL	C57BL	21/24	88	128	ND	ND	ND

*ND = Not done. Statistical analysis: groups 5 + 8 versus 6 + 7: X^2 (corrected) = 5.3; PL 0.05; group 8 versus control group 9: X^2 (corrected) = 10.9; $P < 0.01$.

The (C57BL × BALB/c)F_1 hybrid hosts developed lymphoid tumors with about the same incidence in all treatment groups (table 2). As in the previous experiment, many of the tumors were of lymphoid cell donor origin. A comparison of the average latent periods to death in the 4 groups suggests that the genetic origin of the thymus graft and that of the infected thymocytes both influence susceptibility. The latent period was shortest in the groups bearing a C57BL graft, longest when both graft and cells were derived from BALB/c donors.

In the experiment using the C57BL × C3H strain combination, the most striking observation was the influence of C3H genotype of lymphoma outcome (table 2). A control group of (C57BL × BALB/c)F_1 hybrid hosts had been sent up to ascertain the potency of the virus preparations used. By comparison with these controls, all groups involving (C3H × C57BL)F_1 hybrid animals as hosts showed considerable inhibition of lymphoma development, particularly when the thymocytes used were of C3H origin. The genotype of the thymus grafts appeared to influence latent period, but had little relationship to incidence. None of the tumors in groups 5 to 8 were of C3H genetic origin when tested by transplantation assay.

DISCUSSION

The transplantation behavior of many of these tumors is that of the strain of the thymus cell donor. The fact that thymic lymphoid cells infected *in vitro* with RLV may persist in the grafts to give rise to lymphomas identifies these cells as the target cells for the neoplastic transformation induced by the virus.

The system described makes possible the experimental study of the contribution of various factors to susceptibility to viral leukemogenesis. Strain, age, sex, physiologic state, and other parameters which influence susceptibility may be independently varied for the three components of the system: the thymic lymphoid target cells, the nonlymphoid—presumably reticular-epithelial component of the thymus, and the systemic host environment.

In the experiments reported here, it is evident that thymic lymphoid cells infected by RLV *in vitro* can yield lymphomas, but that the host and thymic graft environment are also of considerable importance in determining the eventual tumor yield. We are currently investigating the capacity of nonthymic lymphoid cells to undergo neoplastic transformation when incubated with RLV *in vitro* and injected into an appropriate thymic graft. It should also be possible to utilize the system for studies on a cellular level of the various factors involved in virus-host interactions, notably those related to the differences in host range exhibited by different leukemogenic viruses (*9*), and possibly as the basis for a combined *in vitro-in vivo* assay for the virus.

SUMMARY

Cells from the thymus or peritoneal exudates of one parental donor strain, after infection with a leukemogenic virus *in vitro*, were injected into thymus grafts from another parental strain that had been implanted intrarenally into thymectomized, irradiated F_1 hybrid host mice. Lymphoid tumor incidence and latent period with this procedure were comparable to those resulting from direct injection of RLV into intrarenal thymic grafts in similar hosts. On the basis of transplantation behavior, tumors resulting from direct RLV injection were of graft-donor or, less often, of host derivation, as were those which developed after injection of peritoneal cells infected with RLV *in vitro*, with rare exceptions. In contrast, most of the tumors resulting from the injection of neonatal thymocytes, infected with RLV *in vitro*, were of cell-donor origin; such tumors must have developed from the injected thymic lymphoid cells, which are thus identified as the target cells for the neoplastic transformation induced by the virus. It was also established, however, that host genotype and thymic graft viability exert a strong influence on tumor incidence. The significance and interaction of other factors in determining susceptibility to murine viral leukemogenesis should also be amenable to study with this experimental system.

REFERENCES

(*1*) Gross, L.: Oncogenic Viruses. Pergamon Press, New York, 1961.
(*2*) Miller, J. F. A. P.: Etiology and pathogenesis of mouse leukemia. Advances Cancer Res 6: 291–368, 1961.
(*3*) Lieberman, M., and Kaplan, H. S.: Leukemogenic activity of filtrates from radiation-induced lymphoid tumors of mice. Science 130: 387–388, 1959.
(*4*) Lieberman, M., Haran-Ghera, N., and Kaplan, H. S.: Potentiation of virus leukaemogenesis in C57BL mice by X-irradiation or urethane. Nature (London) 203: 420–422, 1964.
(*5*) Haran-Ghera, N., Lieberman, M., and Kaplan, H. S.: Direct action of a leukemogenic virus on the thymus. Cancer Res 26: 438–442, 1966.
(*6*) Kantoch, M., Warwick, A., and Bang, F. B.: The cellular nature of genetic susceptibility to a virus. J Exp Med 117: 781–798, 1963.
(*7*) Miller, J. F. A. P.: CIBA Symposium: Tumor Viruses of Murine Origin. London, J. A. Churchill, 1962, pp 262–283.
(*8*) Metcalf, D., and Wakonig-Vaartaja, R.: Stem cell replacement in normal thymus grafts. Proc Soc Exp Biol Med 115: 731–735, 1964.
(*9*) Kaplan, H. S.: The role of radiation in experimental leukemogenesis. Nat Cancer Inst Monogr 14: 207–220, 1964.

DISCUSSION

Dr. Metcalf: Dr. Lieberman and Dr. Kaplan, I would like to comment on this beautiful system. Some years ago we did an experiment essentially similar to this confirming what you have just shown. We used AKR F_1 hybrid intact animals and into these animals we injected intramuscularly into the thigh, AKR parental type thymic cells from mice at various stages in the preleukemic period. Subsequently, at least some of these recipient animals developed thymic lymphoma which we showed had been derived from the injected cells. So thymic cells probably carry the virus, can seed in the thymus and there become neoplastic.

However, we also could show this with spleen cells (Metcalf, D., Cancer Res. 23: 1774, 1963). Have you tried suspensions of spleen cells, *i.e.*, infecting them *in vitro*, to see whether they would also produce the same results when injected into the thymus?

Dr. Lieberman: We have infected various types of cells *in vitro* and injected them into thymus grafts, but have no results yet.

Chairman Furth: Macrophages carried the virus. They never produced anything, did they, themselves?

Dr. Lieberman: The "macrophages" we used were in fact a mixture of peritoneal cells. They carried the virus into the graft but, with 2 or 3 exceptions, did not themselves undergo neoplastic transformation.

Dr. Barski: Have you tried to maintain your thymocytes for longer periods or to cultivate them?

Dr. Lieberman: Not in this experiment. We tried in other experiments to maintain lymphocytes *in vitro*, but were unsuccessful.

Dr. Barski: Are they not growing or are they not carrying the virus? What is happening?

Dr. Lieberman: We have so far been unable to keep thymic lymphocytes alive *in vitro* for more than a few days. They gradually die out, whether or not they have been infected with radiation leukemia virus.

Dr. Upton: There is growing evidence that hematopoietic cells of the C57BL phenotype, or those derived from other H^{-2b} H^{-2b} genotypes, may be antigenic to F_1 recipients. I refer specifically to recent work by G. Cudkowicz *et al.*, at Oak Ridge (*see* Immunology 7: 291, 1964). For this reason one must view with a little reservation the behavior of C57BL grafts in F_1 hybrid recipients. One should not conceive of the C57BL and the BALB/c as having an equal advantage for growth in the (BALB/c × C57BL)F_1.

Dr. Tennant: I found the data extremely interesting, particularly in view of some experiments which I have recently completed (J. Nat. Cancer Inst. 36: 599–606, 1966). In these experiments, thymus cells from baby BALB/c mice were admixed with the B/T-L virus *in vitro*, and the admixture injected intraperitoneally into totally resistant C57BR/cd infants after 30 minutes at room temperature. The results were as follows: 30% of the C57BR/cd mice came down with lymphoid leukemia; these leukemias were transplantable to C57BR and not to BALB/c weanlings.

I made extracts of these C57BR leukemias and the virus went in both the C57BR and BALB newborns. This virus I have called BR-th/T-L, meaning it is of C57BR origin but was induced by the help of the added thymus. This leukemia has since been passed as cell-free filtrate in C57BR through four passages. It now goes 100% in C57BR *and* in BALB, and it also goes in the C58 strain, which shares the $H\text{-}2^k$ allele with C57BR and which was also originally resistant to the B/T-L virus. This is indicative to me of an alteration in the virus due to forced passage through a strain previously totally resistant.

Dr. Ioachim: I would like to report on our approach to try to distinguish which cells of the grafted thymus are effective in restoring sensitivity to leukemia in thymectomized animals. We did this experiment in association with Dr. Furth, and we found a way to separate lymphocytes from reticular cells of the thymus by growing thymuses in tissue cultures. The lymphocytes die out in a few days, so that after about 3 weeks of culture lymphocytes usually are no longer present. The culture is an almost pure monolayer of reticular cells.

These cells, grafted in thymectomized rats injected with virus at birth, were able to restore leukemia. At about the common average latency for viral leukemia, some animals had the generalized disease. More interesting was that they had, in addition, a lymphoma at the site of injection. The injected cells were isolated cells cultured *in vitro*, but after being grafted they aggregated and formed a structure similar to the thymus. In most cultures, the cells grew in a meshwork made up of elongated, plump cells that were considered reticular thymic cells. Among and merging with them were islands of polyhedral cells growing in a pure epithelial pattern.

The graft at the site of injection, by the time the animal had leukemia, had a core of epithelial-reticular cells and many leukemic lymphoblasts about them. This core of cells displayed glandular-like and tubular-like structures and it was difficult to determine if they were neoplastic. However, the cells around them were leukemic lymphoblasts.

What the role of the epithelial-reticular cells in inducing leukemia is constitutes an essential question. The available data are insufficient to answer the question whether these cells become neoplastic themselves or whether they just provide the proper environment to induce leukemogenesis with lymphocytes from the host.

Chairman Furth: Did anybody present convincing evidence that there is a leukemia inducer (in the presence of virus) in an organ other than the thymus?

Dr. Lieberman: We have no such evidence.

Dr. Siegler: Dr. Rich and I would like to confirm the interesting studies that you and Dr. Kaplan reported last year at the cancer meeting (Proc. Amer. Assoc. Cancer Res., 6: 40, 1965) in which you inoculated virus directly into one of the thymuses and found that the tumor developed in the inoculated thymus with a greater incidence than it did in the uninoculated thymus. We have now done this in a large series of animals, and we can conclude that the leukemogenic effect of the virus is direct.

Dr. Wheelock: I seem to have missed the evidence you presented that these thymocytes and peritoneal cells were actually infected. You certainly inoculated them *in vitro*, but they may have carried the virus with them into the graft. And I think some of your data showed that the virus inoculated alone produced similar tumors. What evidence is there that these cells were actually infected?

Dr. Lieberman: When lymphomas were induced by inoculation of either free RLV or RLV-incubated peritoneal cells into the grafts, their transplantation behavior indicated that they were of graft or, more rarely, of host origin. However, the tumors which arose after inoculation of RLV-incubated thymocytes were, in most cases, of thymocyte donor genotype. It is this evidence which leads us to conclude that the thymocytes were actually infected with the virus and underwent neoplastic transformation.

Dr. Latarjet: This is a really beautiful system and there are other interesting questions that you probably have started to study, for example, the question of the kinetics of some events. When you inject the virus directly into the graft, since the graft is progressively repopulated by the host cells, you may get some ideas as to the time required for the establishment of malignancy. Do you now have some really very fundamental ideas on this point?

Dr. Lieberman: We have information bearing on this question, which will be presented in detail elsewhere. Briefly, we injected RLV into thymus grafts of parental genotype at increasing time intervals after their implantation in F_1 hybrid hosts. Transplantation behavior of the ensuing lymphomas indicated a gradual shift from parental to hybrid genotype. This suggests that RLV acts almost immediately to promote the continued replication, and thus to prevent the normal disappearance of donor cells from such grafts.

Chairman Furth: That is not mutually exclusive, is it? If one is made neoplastic it doesn't exclude the possibility that the other becomes neoplastic, so theoretically you may get, and in fact can find, mixed types.

Dr. Lieberman: Theoretically, yes.

Dr. Sinkovics: Have any of you who are inoculating leukemic lymphocytes ever observed any autoimmune manifestation associated with leukemia, like Coombs'-positive hemolytic anemia or runting, in the recipient?

As you know, a few years ago we observed a mouse lymphoma and we thought that the cell population consisted of at least two types of lymphoid cells, one causing leukemia and the other causing runt disease with Coombs'-positive hemolytic anemia in recipients of the same mouse line. In chronic human lymphocytic leukemia such an association of leukemia and autoimmunity is quite common.

Dr. Lieberman: We have not observed anything of the kind.

Chairman Furth: Has anybody ever observed Coombs' positive anemia?

Dr. Siegler: We have tested leukemic animals with Coombs' antiserum and have never observed a positive response.

Dr. Chieco-Bianchi: Dr. Lieberman, which technique of transplantation did you use to test the genotype? Did you count cells?

Dr. Lieberman: We made a suspension of the tumor and injected a known number of cells intraperitoneally into mice of the appropriate genotype.

Dr. Chieco-Bianchi: As shown in the table, when the virus is injected directly into the graft, most of the lymphomas are of BALB parental genotype. How do you explain this? Usually the thymic graft is repopulated by the host lymphocytes.

Dr. Lieberman: At the time RLV is injected into the graft, the thymocytes present are still, in most cases, of BALB/c graft genotype. These cells are infected by the virus and eventually become neoplastic. In some instances, the tumor turns out to be of F_1 host origin; here it is probable that the graft had already been repopulated by host cells at the time of virus inoculation.

Chairman Furth: It would require a special type of assay, wouldn't it?

Dr. Lieberman: Yes. The transplantation assay we have used would not enable us to establish the simultaneous presence, in a lymphoma, of cells of both graft and host origin.

Prelymphomatous or Preleukemic State in Mice: Relation to Human Preleukemia [1, 2]

MATTHEW BLOCK, *Ph.D.*, *M.D.*, *Department of Medicine, University of Colorado Medical Center, Denver, Colorado 80220*

IN 1953 Dr. Jacobson, Dr. Bethard, and I published a paper on the preleukemic state in man, based upon fortuitous observations on 12 patients (*1*). The blood, marrow, and, in some cases, liver and spleen of these patients had been studied serially from the time they presented with a variety of hematologic abnormalities until they developed acute leukemia. These initial observations have since been supplemented by analysis of about another 50 patients (*2*), and similar observations on the preleukemic state have been made by many other authors (*3–13*).

When first seen, all patients had a maturation arrest and aplasia of neutrophil precursors in the marrow and a neutropenia. Erythroblasts and megakaryocytes in the marrow were usually normal. Some patients had a decrease in erythroblasts and megakaryocytes and a pancytopenia, simulating the findings in aplastic anemia. Other patients had a marked hyperplasia of erythroblasts with a mild erythroblastemia resembling erythroleukemia or sideroblastic anemia. No patient showed the erythroblastic metaplasia of liver and spleen, the extreme erythroblastic hyperplasia of the marrow, severe erythroblastemia, and atypical erythroblasts of erythremic myelosis (Di Guglielmo's disease). Regardless of the state of erythroblasts or megakaryocytes, it must be emphasized that, when the patients were first seen, there was a maturation arrest and aplasia of neutrophil precursors in the marrow and neutropenia.

Over a period of time, varying from 6 days to several years, the granulocyte precursors increased, with progressive loss of maturation until stem cell or acute granulocytic leukemia developed. The peripheral blood, liver, and spleen became recognizably leukemic only after overt leukemia

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by an institutional cancer grant of the American Cancer Society, Inc., to the University of Colorado; Fluid Research Fund of the University of Colorado Medical School; and a grant from the Hematology Research Foundation, Chicago, Ill.

was found in the marrow. Other investigators have confirmed these observations except Danopoulos (*14*) who has demonstrated the histopathologic findings of acute leukemia in the spleen prior to its occurrence in the marrow. A preleukemic state has not been recognized in chronic granulocytic or chronic lymphocytic leukemia in man.

Systematic investigation of the preleukemic state in man was impossible for two reasons. First, we had to rely upon fortuitous discovery of patients who did not have acute leukemia but who would subsequently develop the disease. Second, it was impossible to obtain as much histopathologic material as desired because of danger and discomfort to the patient. Accordingly, our attention turned to the mouse where it was alleged that acute leukemia could be induced in a consistent and predictable manner. The histopathology of the preleukemic state could then be systematically analyzed during the interval between the first application of the leukemogenic agent and first sign of acute leukemia. Therefore, the "preleukemic" state was investigated in the DBA mouse painted with methylcholanthrene (*15*), in the C57BL/6 mouse irradiated by Kaplan's technique (*16*), and in the C3Hf/Bi mouse inoculated with Passage A virus obtained from Dr. Gross (*17*). The results of this investigation showed that a similar type of disease best designated as thymic lymphoma, at least in terms of nomenclature current in human disease, developed in the C57BL/6 and C3Hf/Bi mice and an entirely different type in the DBA mouse.

MURINE THYMIC LYMPHOMA

Development of Thymic Lymphoma in C57BL/6 and C3Hf/Bi Mice

Atrophic phase.—In both the virus-induced and radiation-induced thymic lymphomas, lymphocytic depletion of the thymus always preceded the development of overt malignancy (*16*, *17*). In the C3Hf/Bi mice treated with Gross' Passage A virus, the atrophy was focal with large residual areas of normal thymus. In irradiated C57BL/6 mice, the entire thymus was uniformly depleted of lymphocytes. In either case, the atrophic tissue was composed almost exclusively of reticular cells.

Regenerative phase.—The next step in the development of thymic lymphoma was the formation of foci of large free cells. The cells in any focus were identical to each other and were of three general types: large lymphocytes, medium lymphocytes, and reticular lymphocytes, cells intermediate in morphology between reticular cells and medium and large lymphocytes. Regardless of the cell type, mitoses and degenerating cells were abnormally numerous. In the C3Hf/Bi mice, these foci were always in atrophic areas and never in residual normal tissue. In the C57BL/6 mice, these cells appeared diffusely through the cortex about 6 weeks after radiation when repletion of the radiation-induced lymphocytic cortical atrophy was complete.

We have considered these regenerative foci of reticular or medium or large lymphocytes to be the end of the premalignant phase and the first evidence of thymic lymphoma for the following reasons. There is no means of distinguishing these foci from obvious lymphoma *in situ* except that they immediately precede and also are smaller, especially in the C3H mouse, than obvious lymphoma which subsequently develops in the thymus. The cellular composition of the regenerative foci is extremely abnormal and unlike anything found in normal mice. It is admitted that isolated, single cells similar to those seen in the regenerative foci occur in the thymus in embryonic life (*18–21*), during reconstitution of a normal thymus after lymphocytic depletion (*22*, *23*), and even in the normal postnatal thymus (*17*, *24*, *25*). However, in these instances of nonmalignancy, islands of monomorphous cells with numerous dying and dividing cells did not occur. Medium and then small lymphocytes appeared among the large lymphocytes, which resulted in a more pleomorphic tissue than found in the lymphomatous C57BL/6 or C3Hf/Bi mice.

Progressive development of thymic lymphoma.—The regenerating lymphomatous foci thereafter rapidly enlarge. The cells in the lymphomatous tissue are so packed together and monomorphic that this tissue resembles an epithelial syncytium. A "starry sky" appearance is produced by the large number of dying cells, a phenomenon also found in man with highly malignant lymphomas (*26*), typified by Burkitt's South African lymphoma (*27*, *28*), and in chemically induced murine thymic lymphoma (*29*).

Many animals succumb to the effect of the expanding thymic tumor on adjacent mediastinal structures prior to formation of extrathymic lymphoma. Very few survive long enough to develop overt leukemia with marrow and peripheral blood involvement. Thymic lymphoma may, therefore, be considered to be a generalized disease first appearing in the thymus, subsequently in other tissues, and eventually in the blood, at which time the animals are leukemic.

Mechanism of Development of Thymic Lymphoma

In the C57BL/6 and C3Hf/Bi mice, depletion of thymic lymphocytes always preceded development of thymic lymphoma. A similar lymphocytic depletion has been shown to precede development of 1) spontaneous lymphoma in AK mice (*30*), 2) Moloney virus-induced lymphoma in BALB/c mice (*31*), 3) radiation-induced lymphoma in C57 mice (*16*, *32*), and 4) 7,12-dimethylbenz[*a*]anthracene-induced (*29*) and urethan-induced (*33*) thymic lymphoma in mice.

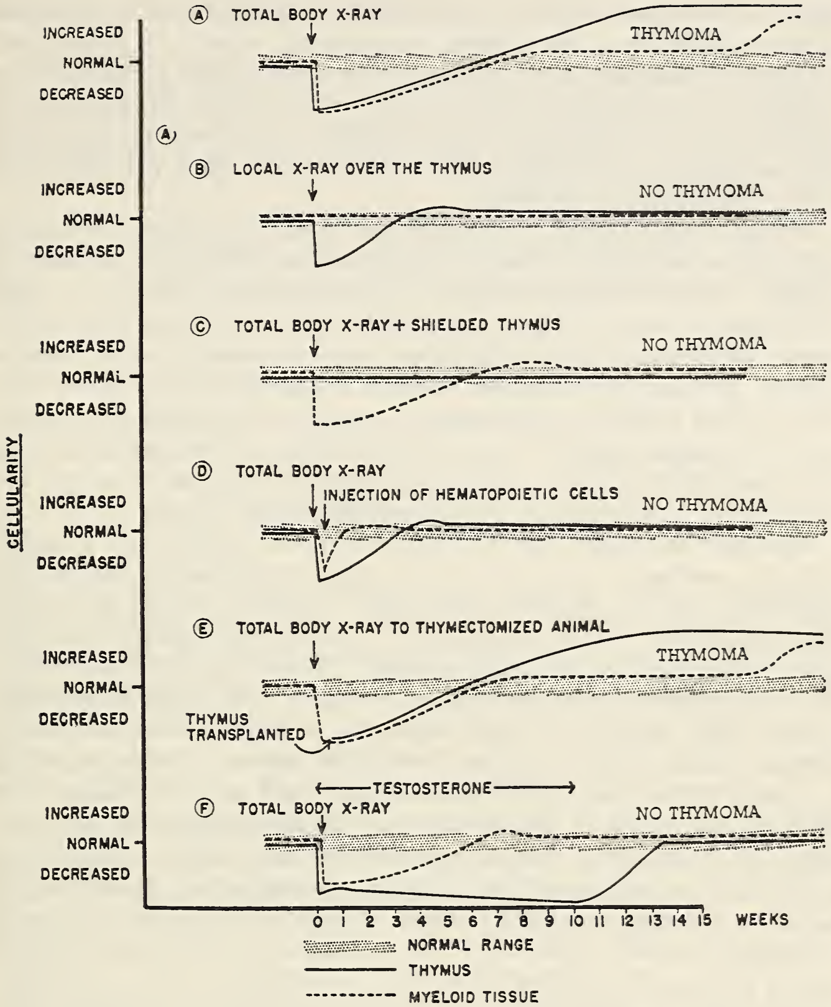
The thymic factor may be further analyzed in C57BL/6 mice treated with testosterone, which is known to inhibit radiation-induced thymic lymphoma (*16*, *34*, *35*). Testosterone causes lymphocytic depletion of the normal thymus and has little discernible effect on extrathymic lymphatic tissues or marrow (*16*, *36*). Testosterone has no effect on karyorrhexis and lymphocytic depletion in C57BL/6 mice exposed to radiation known to induce thymic lymphoma. However, testosterone inhibited both the regeneration occurring in the first 6 weeks and the malignant proliferation beginning 6 weeks after radiation. When thymic lymphoma developed in irradiated mice, the thymus of the mice radiated and treated with testosterone was still composed almost entirely of reticulum cells with only a few scattered lymphocytes. In contrast, development of thymic lymphoma was not inhibited if testosterone treatment was begun 3 weeks after radiation, *i.e.*, after repletion of the thymus by lymphocytes had begun.

Therefore, most probably thymic lymphoma developed as a result of an abnormal regeneration in response to depletion of thymic lymphocytes.

The development of thymic lymphoma in thymectomized and irradiated mice subsequently implanted with a normal thymus (*37*) seems to contradict the hypothesis that a thymus must undergo atrophy and subsequent regeneration in order for thymic lymphoma to develop. However, after transplantation, thymic lymphocytes are almost completely destroyed and then regenerate (*38*, *39*). Therefore, the sequence of events (destruction and regeneration of lymphocytes), postulated to occur in the thymus prior to development of thymic lymphoma in the intact irradiated mouse, did occur in the normal thymus transplanted into the irradiated and thymectomized mouse.

An *extrathymic factor* must also be considered. Radiation of the thymus alone does not induce thymic lymphoma even though the radiated thymus undergoes the same karyorrhexis, atrophy, and regeneration seen when the entire mouse is irradiated (*40*). Splenic shielding (*41*), leg shielding (*42*, *43*), and intravenous injection of marrow (*44*) or splenic cells (*45*) prevent thymic lymphoma in irradiated mice without preventing the initial thymic lympholysis and subsequent lymphocytic regeneration. These procedures, however, do modify the environment, especially the myeloid tissue in the spleen and marrow in which thymic atrophy and regeneration occur. Marrow abnormalities resembling those seen after irradiation have been demonstrated in coincidence with thymic regeneration in mice in which thymic lymphoma has been induced by 7,12-dimethylbenz[*a*]anthracene (*29*) and by urethan (*33*). The marrow of virus-induced thymic lymphoma has not been analyzed in sufficient detail to evaluate its role in thymic lymphoma. If testosterone is discontinued 10 weeks after thymic regeneration, thymic lymphoma does not develop. If treatment with testosterone is begun 3 weeks after radiation, thymic lymphoma develops (*36*). Therefore, the thymic and extrathymic factors must not only be present but must *coexist* (*16*).

The evidence, therefore, strongly suggests that development of thymic lymphoma depends on coexistence of a thymic factor and an extrathymic factor. The former is a depletion of thymic lymphocytes leading to thymic atrophy which induces a neoplastic regeneration. Regeneration is neo-



Text-figure 1.—Mechanism of development of thymic lymphoma in mice exposed to radiation. Reproduced in modified form from *Cancer Research* 21: 463–473, 1961, with the consent of the editor.

plastic only if it occurs when myeloid tissue is damaged. Removal of the thymic or extrathymic factor or temporal dissociation of one from the other inhibits the development of thymic lymphoma.

Relevance of Development of Murine Thymic Lymphoma to the Preleukemic State in Man

Our purpose in investigating murine thymic lymphoma was to extend observations originally made on the preleukemic state in man. Obviously, there is little direct similarity between thymic lymphoma in the mouse (which originates in the thymus, subsequently extends to extrathymic lymphatic tissue, and only very late in the course of the disease involves the marrow and peripheral blood) and acute stem cell or acute granulocytic leukemia in man (which is first demonstrable in the marrow, next the blood, and subsequently in lymphatic tissue, and in which there is little, if any, thymic involvement at any time).

In man, acute lymphatic leukemia may develop in patients who have lymphosarcoma or giant follicular lymphoma, the incidence of the leukemia varying roughly with the duration of the lymphosarcoma (*46*). When the lymphosarcoma is first recognized, the disease is largely restricted to lymph nodes with usually little, if any, involvement of marrow, liver, or spleen. In these patients, overt leukemia is preceded by lymphatic metaplasia of the marrow. This sequence of events is simulated by murine thymic lymphoma only in that the first evidence of malignancy is extramedullary (thymic in the mouse and primarily nodal in man) and that medullary and peripheral blood diseases occur relatively late.

Especially in infants and children, rarely are symptoms of a rapidly enlarging mediastinal mass the major and perhaps the sole abnormality detectable when the patient is first seen. In these patients the mass is almost invariably nodal and not thymic. The thymus is usually so atrophic that it is hard to find on mediastinotomy. It is unlikely the mediastinal mass is thymus so altered by malignancy that it is no longer recognizable.

Although I have so far emphasized the lack of obvious relationship between murine thymic lymphoma and acute leukemia in man, especially in regard to the preleukemic state, there is still some similarity. The premalignant phase in murine thymoma is characterized by an initial lymphocytic depletion, due to acute karyorrhexis, followed by atrophy and then by an ungoverned regeneration. In our human material (*1*, *2*), we have not detected the initial destructive phase. It has been impossible to determine whether this failure has been because such a phase does not exist or because of the difficulties in studying this phase in man. However, a similar acute karyorrhexis occurred immediately after irradiation prior to development of acute stem cell and granulocytic leukemia induced in the Japanese at Hiroshima and Nagasaki by an atomic bomb explosion, and also to a lesser extent in patients with polycythemia vera treated by radiation, especially radiophosphorus (*47*).

The atrophic stage and subsequent abnormal, uncontrolled regeneration of lymphocytes in murine lymphoma are similar to the maturation arrest and aplasia of granulocytes and subsequent abnormal regeneration of stem cells and immature granulocytes in human acute leukemia. Apparently the same basic phenomena, damage to cells, atrophy, and attempt at regeneration culminating in malignant, uncontrolled growth, occur in both species, in the thymus of the mouse and in the marrow in man.

THYMIC-INDEPENDENT HEMATOPOIETIC MALIGNANCIES

A generalized, extremely anaplastic lymphosarcoma (*15*) developed in the DBA mouse painted with methylcholanthrene. The initial effect of the methylcholanthrene was to stimulate granulocytopoiesis in the marrow and splenic red pulp and to initiate granulocytopoiesis in hepatic portal tissue. The number of erythroblasts and megakaryocytes in the splenic red pulp and the amount of splenic white pulp decreased with progressive proliferation and subsequent progressive maturation arrest of granulocyte precursors in the splenic red pulp. The lymphosarcoma apparently developed in the anaplastic tissue resulting from the progressive maturation arrest of the granulocytopoietic tissue in the spleen and portal tissue. I could not exclude the possibility that the cells composing the lymphosarcoma were the stem cells for the granulocytic tissue. Lymphosarcomatous tissue also formed at the same time in lymph nodes and kidney. The marrow remained granulocytopoietic, the peripheral blood did not become leukemic, and the thymus remained atrophic throughout the entire course of the disease.

This sequence of events is similar to what occurs in the spleen of patients with polycythemia vera who develop acute granulocytic leukemia after repeated injections of radiophosphorus (*47*). In both man and the DBA mouse, the malignancy arises in splenic red pulp which is the site of an intense myeloid metaplasia. In chronic granulocytic leukemia in man, it is not uncommon to find replacement of granulocytic tissue by cells indistinguishable from those in an anaplastic lymphoma (*47–50*).

Granulocytic leukemia, induced in a predictably high percentage of RF mice exposed to 350 r of whole-body X radiation (*51*), closely resembles, in both its tissue and peripheral blood manifestations, acute or subacute granulocytic leukemia in man. The preleukemic stage has not been studied.

Granulocytic leukemia is induced in thymectomized mice and rats by techniques which ordinarily induce thymic lymphoma in corresponding intact animals (*52–55*) and in rats treated by gastric instillation of methylcholanthrene (*56*, *57*). Siegler and Rich have described the development of granulocytic leukemia in splenic red pulp, next in the liver, circulation and lymph nodes, and finally in the marrow in mice under these circumstances (*53*). Paralleling the preleukemic state in human acute leukemia, a peripheral blood neutropenia was found prior to development of overt leukemia. Further investigation of the preleukemic phase in murine granulocytic leukemia should yield information more directly applicable to human leukemia than has been the case with murine thymic lymphoma.

SUPPRESSION OF NORMAL HEMATOPOIETIC TISSUE

All investigations on the preleukemic state and on treatment of leukemia have focused on the leukemic tissue and not on normal hematopoietic tissue. In man, the overwhelming majority of patients with acute and chronic leukemia die because of decreased production of red cells, granulocytes, mononuclears, platelets, and antibodies, sometimes compounded by premature destruction or hemorrhage of these cells. Death due to proliferation of leukemia tissue *per se* is relatively rare. The naive concept that proliferation of leukemic tissue simply displaces normal tissue is not tenable. It is important to explain the mechanism by which normal hematopoiesis is suppressed, not in vague terms such as displacement of normal tissue by leukemic tissue, but rather in terms of the production kinetics (replications of stem cells, influx, maturation time, cell division, and ineffective production) and maturation of the cells in the hematopoietic tissues (*58*, *59*).

SUMMARY

The preleukemic state in human acute leukemia has been described. In order to extend observations made in man on the preleukemic state, the development of thymic lymphoma in irradiated C57BL/6 mice and in C3Hf/Bi mice inoculated with Gross' Passage A virus was investigated. The development of thymic lymphoma in these 2 strains is dependent on the coexistence of a thymic factor (depletion of thymic lymphocytes in lymphocytes and thymic atrophy leading to malignant degeneration) and an extrathymic factor (abnormal myeloid tissue). Although differing in detail, the premalignant phases of murine thymic lymphoma and of human acute leukemia are basically similar in that both represent an abnormal attempt at regeneration of a depleted cell type or lineage. The premalignant phase of generalized lymphosarcoma induced by methylcholanthrene in DBA mice is described. This and the granulocytic leukemia induced in RF mice and in thymectomized mice and rats resemble human granulocytic leukemia. A very important aspect of the preleukemic state, the mechanism by which normal hematopoiesis is suppressed, has been neglected and deserves attention.

REFERENCES

(*1*) Block, M., Jacobson, L. O., and Bethard, W. F.: Preleukemic acute human leukemia. JAMA 152: 1018–1028, 1953.

(*2*) Block, M.: Histopathology of the preleukemic state in humans. Boston, Sixth Congress Meeting of Society of Hematology, Aug. 27–Sept. 1, 1956, pp 195–196.

(*3*) Paraf, A.: Anémie pernicieuse avec mégaloblastose médullaire résistante à l'hépatothérapie. Terminaison en leucose aiguë. Rev Hemat 4: 655–669, 1949.

(*4*) André, L., Marty, J., Rispe, R., and Morichau-Beauchant, J.: Sur un cas de leucémie aiguë précédé de neutropénie pendant dix-huit mois. Bull Soc Med Hop Paris 69: 417–421, 1953.

(*5*) Dreyfuss, B., and Bessis, M.: Leucémies aiguës précédées d'une aplasie myeloïde. Sang 25: 787–797, 1954.

(*6*) Meacham, C., and Weisberger, A.: Early atypical manifestations of leukeumia. Ann Intern Med 41: 780–797, 1954.

(*7*) Bernard, J., and Boiron, M.: Les anémies préleucoblastiques de la leucose aiguë. Sang 25: 797–825, 1954.

(*8*) Williams, M. J.: Myeloblastic leukemia preceded by prolonged hematologic disorder. Blood 10: 502–509, 1955.

(*9*) Verloop, M. C.: Anaemia in systemic disease. Acta Med Scand 151: 367–380, 1955.

(*10*) Goldschmidt, L.: Die partielle and die Fötale Haemocythophtise und thre Bedeutung als Vorstadium der Leukämie in Kindesalter. Ann Paediat 184: 26–62, 1955.

(*11*) Bottiger, L. E., and Hellström, O.: Erythroblastomatosis. Acta Haemat 16: 329–337, 1956.

(*12*) Koeffler, H.: Zur Pathogenese, Klinik und differential Diagnose symptomatischer aplasticher Anämieformen mit der leukotischem Endbild. Med Klin 55: 1394–1397, 1960.

(*13*) DeGowin, R. L.: Benzene exposure and aplastic anemia followed by leukemia 15 years later. JAMA 185: 748–751, 1963.

(*14*) Danopoulos, E., and Angelopoulos, B.: Der extramedulläre Beginn der akuten Leukämien. Med Klin 49: 1947–1950, 1954.

(*15*) Block, M.: The presarcomatous stages of methylcholanthrene induced lymphosarcoma in the DBA mouse. Proc Amer Assoc Cancer Res 1: 6, 1954.

(*16*) Wasi, P., and Block, M.: The histopathologic study of the development of the irradiation-induced leukemia in C57BL mice and of its inhibition by testosterone. Cancer Res 21: 463–473, 1961.

(*17*) Goodman, S. B., and Block, M.: The histogenesis of Gross's viral induced mouse leukemia. Cancer Res 23: 1634–1640, 1963.

(*18*) Maximow, A.: Untersuchungen über Blut und Bindegewebe. II. Über die Histogenese der Thymus bei Saügetieren. Arch Mikr Anat 74: 525–621, 1909.

(*19*) ———: Bindegewebe und Blutbildende Gewebe. *In* Handbuch der Mikroskopischen Anatomie des Menschen (Möllendorf, W. V., ed.). Berlin, Springer, vol I, part 1, 1927.

(*20*) Block, M.: The blood forming tissues and blood of the newborn opossum (Didelphys Virginiana). I. Normal development through about the one hundredth day of life. Ergebn Anat Entwicklungsgesch 37: 237–366, 1964.

(*21*) Ackerman, G. A., and Knouff, R. A.: Lymphocyte formation in the thymus of the embryonic chick. Anat Rec 149: 191–215, 1964.

(*22*) Murray, R. G.: Histopathology of irradiation from external and internal sources. *In* The Thymus. National Nuclear Energy Series, Manhattan Project Technical Series, Division IV. Plutonium Project Record Vol. 22, IX. New York, McGraw-Hill Book Co., Inc., 1948, chapt 9.

(*23*) Dougherty, T. F., and White, A.: Functional alterations in lymphoid tissue induced by adrenal cortical secretion. Amer J Anat 77: 81–116, 1945.

(*24*) Saint Marie, G., and Leblond, C. P.: Cytologic features and cellular migration in the cortex and medulla of the thymus in the young adult rat. Blood 23: 275–299, 1964.

(*25*) Murray, R. G., Murray A., and Pizzo, A.: The fine structure of the thymus of young rats. Anat Rec 151: 17–30, 1965.

(*26*) Diamandopoulos, G. Th., and Smith, E. B.: Phagocytosis in reticulum cell sarcoma. Cancer 17: 329–337, 1964.

(27) Burkitt, D., and O'Conor, G. T.: Malignant lymphoma in African children. I. A clinical syndrome. Cancer 14: 258–269, 1961.

(28) Booth, C. C.: A case of African lymphoma. Brit Med J 1: 294–299, 1964.

(29) Rappaport, H., and Baroni, C.: A study of the pathogenesis of malignant lymphoma induced in the Swiss mouse by 7,12-dimethylbenz[*a*]anthracene injected at birth. Cancer Res 22: 1067–1074, 1962.

(30) Arensen, K.: Preleukemic and early leukemic changes in the thymus of mice; a study of the AKR/O strain. Acta Path Microbiol Scand 43: 350–364, 1958.

(31) Dunn, T. B., Moloney, J. B., Green, A. W., and Arnold, B.: Pathogenesis of a virus-induced leukemia in mice. J Nat Cancer Inst 26: 189–221, 1961.

(32) Kaplan, H.: Early microscopic diagnosis of lymphosarcoma in situ in thymus of irradiated mice. Fed Proc 19: 399, 1960.

(33) Haran-Ghera, N., and Kaplan, H. S.: Significance of thymus and marrow injury in urethan leukemogenesis. Cancer Res 24: 1926–1931, 1964.

(34) Gardner, W. U.: Ovarian and lymphoid tumors in female mice subsequent to roentgen-ray irradiation and hormone treatment. Proc Soc Exp Biol Med 75: 434–436, 1950.

(35) Kaplan, H. S., and Brown, M. B.: Testosterone prevention of post-irradiation lymphomas in C57 black mice. Cancer Res 12: 445–447, 1952.

(36) Kaplan, H. S., Nagareda, C. S., and Brown, M. B.: Endocrine factors and radiation-induced lymphoid tumors. Recent Progr Hormone Res 10: 293–332, 1954.

(37) Kaplan, H. S., Hirsch, B. B., and Brown, M. B.: Indirect induction of lymphomas in irradiated mice. IV. Genetic evidence of the origin of the tumor cells from the thymic grafts. Cancer Res 16: 434–436, 1956.

(38) Reinhardt, W. O., and Wainman, P.: Effect of thyroidectomy, castration, and replacement therapy on thymus, lymph nodes, spleen in male rats. Proc Soc Exp Biol Med 49: 257–260, 1942.

(39) Carnes, W. H., Kaplan, H. S., Brown, M. B., and Hirsch, B. B.: Indirect induction of lymphomas in irradiated mice. III. Role of the thymic graft. Cancer Res 16: 429–433, 1956.

(40) Kaplan, H. S.: Preliminary studies of the effectiveness of local irradiation in the induction of lymphoid tumors in mice. J Nat Cancer Inst 10: 267–270, 1949.

(41) Lorenz, E., Congdon, C. C., and Uphoff, D.: Prevention of irradiation-induced lymphoid tumors in C57BL mice by spleen protection. J Nat Cancer Inst 14: 291–301, 1953.

(42) Kaplan, H. S., and Brown, M. B.: Further observations on inhibition of lymphoid tumor development by shielding and partial-body irradiation of mice. J Nat Cancer Inst 12: 427–436, 1951.

(43) ———: Protection against radiation-induced lymphoma development by shielding and partial-body irradiation of mice. Cancer Res 12: 441–444, 1952.

(44) Kaplan, H. S., Brown, M. B., and Paull, J.: Influence of bone-marrow injections on involution and neoplasia of mouse thymus after systemic irradiation. J Nat Cancer Inst 14: 303–316, 1953.

(45) Cole, L. J., Nowell, P. C., and Ellis, M. E.: Incidence of neoplasms in mice protected against lethal doses of X-rays by spleen homogenate. Proc Amer Assoc Cancer Res 2: 100, 1956.

(46) Block, M.: Histopathologic basis for evaluation of prognosis and response to therapy of the lymphocytic lymphomas. *In* The Lymphocyte and Lymphocytic Tissues (Rebuck, J., ed.). International Academy of Pathology Monograph. New York, Paul B. Hoeber Inc., Medical Division Harper and Brothers, chapt 17, 1960.

(47) ———: Unpublished data.

(48) Lüscher, M.: Reticulosarkom des Humerus mit Spontanfraktur bei chronischer myeloischer Leukämie. Sch M Wchnschr 90: 983–986, 1960.
(49) Wildhack, R.: Beitrag zu den terminalstadien der chromischen Myelose. Folia Haemat (Frankfort) 5: 349–365, 1961.
(50) Fischer, R., Lorbacher, P., and Schumacher, K.: Uber den terminaben Ansteig der alkahschen Leukocytenphosphatase bei chrohischer Myelose. Klin Wschr 41: 669–670, 1963.
(51) Upton, A., and Furth, J.: The effects of cortisone on the development of spontaneous leukemia in mice and on its induction by irradiation. Blood 9: 686–695, 1954.
(52) Gross, L.: Serial cell-free passage in rats of the mouse leukemia virus. Effect of thymectomy. Proc Soc Exp Biol Med 112: 939–945, 1963.
(53) Siegler, R., and Rich, M.: Pathogenesis of murine leukemia. Nat Cancer Inst Monogr 22: 525–547, 1966.
(54) Metcalf, D., Wiadrowski, M., and Bradley, R.: Analysis of the role of the thymus in leukemia development with the use of thymectomy and thymus grafts. Nat Cancer Inst Monogr 22: 571–586, 1966.
(55) Fiore-Donati, L., Chieco-Bianchi, L., and Tridente, G.: Studies on thymus-dependent mechanisms of mouse leukemogenesis by Graffi virus. Nat Cancer Inst Monogr 22: 587–603, 1966.
(56) Shay, H., Gruenstein, M., Harris, C., and Glazer, L.: Transfer of myelogenous leukemia induced by gastric instillation of methylcholanthrene in Wistar rats. Blood 7: 613–622, 1952.
(57) Shimkin, M. B., Acuff, M., Thatcher, D., and Gruenstein, M.: Descriptive summary on breast tumors and leukemia induced in female Wistar rats receiving gastric instillations of 3-methylcholantrene. Nat Cancer Inst Monogr 22: 33–59, 1966.
(58) Bloom, W., and Fawcett, D.: Blood cell formation and destruction. *In* Textbook of Histology. Philadelphia, W. B. Saunders Co., chapt V, 1962.
(59) Weinstein, I., and Beutler, E., eds.: Anemia. New York, Blakiston Division, McGraw-Hill, 1962.

DISCUSSION

Dr. Dunn: I appreciate very much Dr. Block bringing in the human problem. I am very glad people are looking for preleukemic changes in man. You certainly are under a great many more handicaps, as we all understand, than you are when dealing with the mouse.

I also appreciate Dr. Block pointing out the importance of atrophy as a preneoplastic condition. We have a little game occasionally of maintaining that atrophy is a much more dangerous preneoplastic state than hyperplasia is, although most of the emphasis in the past has been on hyperplasia. Unfortunately, there are a good many very disconcerting exceptions on both sides, but I do think we need to pay particular attention to the atrophic state, whether in the skin, in the adrenal cortex, or other sites, which may precede neoplasia.

Dr. Kirsten: Dr. Henry Kaplan has shown that one has to have both the thymic as well as the bone marrow injury in radiation-mediated leukemogenesis. Dr. Henry Rappaport's work on 7,12-dimethylbenz[*a*]anthracene-induced thymic lymphomas indicated that certain preleukemic changes occur not only in the thymus but also in the bone marrow. Dr. Dunn and Dr. Siegler, what is known about such bone marrow changes in viral leukemogenesis?

Dr. Block: To comment on Dr. Kirsten's remarks: We did study the bone marrow very carefully. There was no difference in the bone marrow of the animals that received total-body irradiation and those that received total-body irradiation and testosterone, and testosterone alone produced no discernible effect on the bone marrow or the peripheral blood.

Dr. Dunn: There seems to be some uncertainty as to who is to reply to this. I must make a confession that I routinely take bone marrow when I am autopsying mice, but actually I seem to get very little information from it. It is only when it is extremely altered that I feel it gives me any information. I take it only to satisfy those human hematologists who are going to ask you what the bone marrow looked like. For my purposes the spleen is generally so much more informative that it is only as an accessory we take bone marrow.

Dr. Siegler: I agree with Dr. Dunn.

Dr. Rappaport: I am interested in your experiment F in which you gave testosterone and were able to prevent regeneration of the thymus in the total-body irradiated animal. How long did you follow these animals? Did they live out their normal lifespan or did they die earlier? Did these animals ever develop granulocytic leukemia?

Dr. Block: Let me answer the last question first. They did not develop granulocytic leukemia. As to the first question, since we sacrificed many of the animals early in the experiment, and all by 3 or 4 months, to study the premalignant phase, we cannot exclude that they would have developed granulocytic leukemia had they lived longer.

Chairman Furth: Bone marrow, to a good extent, is still a forgotten organ. The comment is well taken. The bone marrow deserves more attention because myelogenesis in man is more likely to originate in the bone marrow than in the spleen. I do not know of any proved case of myeloid leukemia (or myelomatosis) originating in the spleen.

Dr. Metcalf: I agree with Dr. Block's comments that the reasons why leukemic animals or patients die are probably not the presence of the neoplastic cells so much as the anemia from the thrombocytopenia and the susceptibility to infections. I would point out, however, there are people examining these questions by testing mice. We described the occurrence of hemolytic anemia in leukemic mice which was not of the Coombs' positive autoimmune type [Wiadrowski M., and Metcalf D., Nature (London) 198: 1103, 1963]. It was related to the total mass of tumor tissue, and it appeared to us that it might be the result of some product released from the lymphoma cell.

We, and others working on this problem, have also showed that immune responses become severely depressed in mice developing leukemia, even in the very early stages, and there are available techniques to investigate this at the single-cell level using the Jerne plaque technique.

Dr. Block: I think this is very important and ought to be followed through not only for red cells, which are relatively easy to investigate, but also for white cells and platelets that are extremely difficult to investigate.

Dr. Fiore-Donati and I discussed this problem. Hematologists for the last 50 years have been trying to tell everyone else that only hematologists can study the marrow. It isn't that difficult to study and I don't know why there is such reticence about studying it. In addition to that, I think that the bone marrows you showed are very clear-cut. The problem is not that anyone can't investigate the marrow, but that nobody tries to do so.

Analysis of the Role of the Thymus in Leukemia Development With the Use of Thymectomy and Thymus Grafts [1, 2]

D. Metcalf, M. Wiadrowski, *and* R. Bradley, *Cancer Research Laboratory, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Victoria, Australia.*

McENDY, Boon, and Furth (*1*) demonstrated that thymectomy carried out on young adult mice of the high-leukemia strain AKR drastically reduced the subsequent incidence of lymphoid leukemia in these mice.

Following the demonstration by Gross (*2*) that a leukemia-inducing virus could be found in leukemic AKR mice, many workers have reported the isolation of identical or related viruses from cases of lymphoid leukemia in mice. This and subsequent related work has made it likely that most lymphoid leukemia in mice is initiated by a group of related viruses that are either in an active form in some strains, *e.g.*, the AKR, or are present as latent infections in other strains in which they may be activated by irradiation or chemical carcinogens. Recently, evidence has been presented (*3*) to suggest that under certain conditions these viruses also may be capable of inducing myeloid leukemia and reticulum cell sarcoma.

It was shown that thymectomy prevented the viral induction of lymphoid leukemia in mice (*4–6*), although it did not appear to prevent multiplication of the virus in the host animal (*7*) or the transmission of virus to succeeding generations (*8*). In virus-infected thymectomized animals, it was noted that a significant incidence of myeloid leukemia and reticulum cell sarcoma occurred late in life (*9, 10*).

Some years ago it was demonstrated that the thymus produces a lymphocytosis-stimulating factor (*11*) and that plasma levels of this factor are elevated in preleukemic mice, in mice with lymphoid leukemia (*12*) and in mice subjected to whole-body irradiation (*13*). It was shown that thymectomy in preleukemic AKR and C58 mice abolished detectable serum

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria.

lymphocytosis-stimulating activity (*12*). It was suggested that a thymic humoral factor might be involved in leukemogenesis and that production of this factor might be excessive in mice in the preleukemic period. More recently it has been demonstrated that the thymus regulates immune responses in lymphoid organs via a humoral factor (*14*) and, further, that lymphoid leukemic cells induced by leukemia viruses contain an antigen foreign to the host (*15*). This has raised another possibility that the thymus may be essential for lymphoid leukemia development because of some immunological facet of the pathogenesis of the disease requiring the mediation of the thymus.

The present experiments were undertaken to investigate: 1) the possible involvement of a thymic humoral factor in leukemogenesis, 2) the possibility that thymectomy may affect leukemogenesis by modifying immune responses, and 3) other aspects of the role of the thymus in leukemia development.

MATERIAL AND METHODS

Mice.—Mice used were males and virgin females of the inbred strains AKR, C57BL, and C3H, maintained in an inbred state in this Institute, and the F_1 strains (AKR × C3H)F_1 and (AKR × C57BL)F_1.

Thymectomy.—Thymectomy was performed on mice 5 to 6 weeks old, with the use of combined Nembutal and ether anesthesia. A midline sternum splitting approach was used, and the thymus was removed by gentle suction. At autopsy the completeness of thymectomy was checked where necessary by microscopic examination of the upper mediastinal tissues.

Thymus grafting.—Unless otherwise specified, all thymus glands grafted were from donors less than 24 hours old. Intact thymus glands were inserted with forceps into pockets prepared in the subcutaneous tissues of recipient animals either in the axilla or over the thoracic wall.

Diffusion chamber grafts.—Diffusion chambers were constructed from Perspex rings, 1.0 cm in external diameter (0.7 cm internal diameter) and 1.5 mm in depth. Millipore membranes (pore size either 0.45 μ or 0.1 μ) were sealed to the upper and lower aspects of the ring with a solution of Perspex in ethylene dichloride. Neonatal thymus glands or spleens with a small volume of Eagle's medium were loaded into polythene cannulae, of a diameter approximating that of a #18 needle. These cannulae had a finely drawn out tip which was inserted into a hole passing through the Perspex ring of the chamber (side wall). The thymus or spleen tissue with tissue culture fluid was forced into the chamber by gentle pressure on a syringe attached to the cannula. The holes in the chambers were sealed with a thick solution of Perspex in ethylene dichloride. As soon as the cement had hardened, the chambers were inserted without further delay into the peritoneal cavity of recipient mice. Control, empty diffusion chambers prepared in this manner were implanted intraperitoneally into mice and checked for cell content at intervals thereafter. Some empty control chambers with 0.45 μ membrane pore size were found to contain mononuclear cells of host origin, but control chambers with membranes of 0.1 μ pore size were invariably found to be cell free.

Hemagglutination titrations.—Mice were injected intraperitoneally with 0.2 ml of 20% sheep red cells and were bled from the tail vessels on days 3, 5, 7, 10, and 14 following immunization. Sera were titrated by using equal volumes of 2% sheep red blood cells and doubling dilutions of serum in 1% rabbit serum/phosphate-buffered saline. Agglutination patterns were read after 2 hours at room temperature.

Histological examination.—When experimental mice were moribund, they were killed with ether, and tissues for histological examination were fixed overnight at 4° C in 10% Zenker's formalin, dehydrated, and embedded in paraffin. Sections were cut at 5 μ and stained with Mayer's hematoxylin and eosin. Reticular tumors were typed according to the classification of Dunn (*16*).

Autoradiography.—Mice were injected intravenously with 5 μc per g tritiated thymidine (14.8 c/mM; Radiochemical Centre, Amersham, England). Blood films were fixed in methanol/acetic acid and covered with Kodak AR10 stripping film. Films were exposed 10 to 200 days, according to individual requirements of the various time points after labeling, and then stained with Giemsa. The techniques used and criteria for cell classification, labeling, and determining percentage labeling have been described elsewhere (*17*).

RESULTS

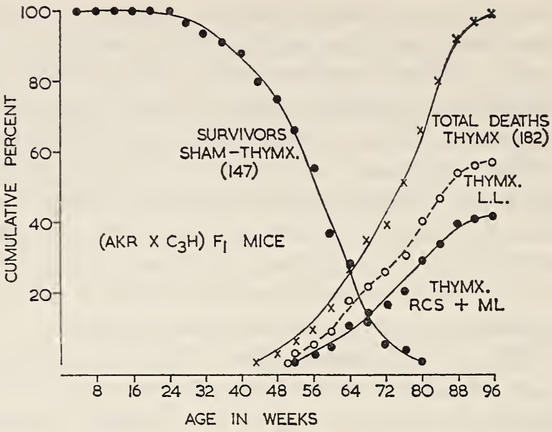
Effect of Thymus and Spleen Tissue in Diffusion Chambers on Leukemia Development

Groups of 5-week-old male and female (AKR × C3H) F_1 mice were thymectomized or sham-thymectomized. Three days later, thymectomized mice were grafted intraperitoneally with: (*a*) empty diffusion chambers; (*b*) diffusion chambers, each of which contained one, 1-day-old, AKR thymus; or (*c*) diffusion chambers, each of which contained one, 1-day-old, AKR spleen. Control thymectomized mice were not grafted.

In an attempt to accelerate leukemogenesis, all thymectomized and sham-thymectomized mice were given 2 injections in the right thigh of 0.3 ml of a filtrate of a freshly prepared 20% saline extract of spontaneous AKR lymphoma tissue, the first injection being 7 days after diffusion chamber grafting and the second injection 1 week later.

The over-all mortality in the thymectomized and sham-thymectomized groups is shown diagrammatically in text-figure 1. No difference in survival times was noted between any of the thymectomized groups, and the data for all 4 groups have been pooled. It will be noted that all sham-

Text-figure 1.—Cumulative mortality in thymectomized and sham-thymectomized (AKR × C3H)F_1 mice. Number of mice in each group is indicated in parentheses. Also shown is cumulative mortality in thymectomized mice from reticulum cell sarcoma (RCS) and myeloid leukemia (ML) (combined) and lymphoid leukemia (L.L.).



thymectomized mice died by 80 weeks of age. In contrast none of the thymectomized mice died before 44 weeks of age, and only 60% died by the time of death of the last sham-thymectomized survivor. All thymectomized mice died by 96 weeks of age.

The incidence of lymphoid leukemia and reticular tumors in sham-thymectomized and thymectomized groups is analyzed in detail in table 1. In the sham-thymectomized mice, the incidence of lymphoid leukemia was 89% (mean age at death, 54 weeks). This incidence and latent period of lymphoid leukemia are similar to those observed previously in untreated F_1 hybrid mice of this type, and it is apparent that the injections of leukemic tissue filtrates had no accelerating effect in the present experi-

Table 1.—Effects of AKR thymus grafts in diffusion chambers on leukemia incidence in thymectomized (AKR × C3H)F_1 mice

Group	Number of mice	Incidence of:			Incidence of deaths from other causes
		Lymphoid leukemia	Myeloid leukemia	Reticulum cell sarcoma	
Sham thymectomy	147	89% (54)*	0%	6% (56)	5% (54)
Thymectomy	43	21% (67)	2% (62)	33% (73)	44% (75)
Thymectomy and empty diffusion chamber	36	11% (71)	3% (74)	33% (71)	53% (71)
Thymectomy and AKR spleen in diffusion chamber	48	15% (68)	8% (64)	35% (75)	42% (70)
Thymectomy and AKR thymus in diffusion chamber	57	12% (67)	2% (86)	46% (76)	40% (79)

*Mean age at death in weeks is indicated in parentheses.

ments. Six percent of the sham-thymectomized mice developed generalized reticulum cell sarcoma of type A. No cases of myeloid leukemia were observed, and 5% of the mice died from other causes, including breast tumors, hepatomas, and generalized infections.

The results from all 4 groups of thymectomized mice were similar. The incidence of lymphoid leukemia was reduced and the latent period to the development of lymphoid leukemia in those mice developing leukemia was considerably longer than in the sham-thymectomized mice. Thymectomized mice with thymus grafts in diffusion chambers did not have any higher or earlier incidence of lymphoid leukemia than thymectomized mice of the other groups. All groups had a low incidence of myeloid leukemia. The most common reticular neoplasm in all 4 thymectomized groups was generalized reticulum cell sarcoma. In approximately half the cases, this was of classical type A, but in the other half a mixed type of reticular tumor developed with a high content of apparently nondividing lymphoid cells. All have been grouped together in the table as reticulum cell sarcoma. In the thymectomized mice moribund from other causes, the most common apparent cause was a generalized low-grade infection involving the liver, spleen, and lymphoid organs which were enlarged and infiltrated by pleomorphic aggregates of lymphoid, plasma, and reticulum cells and occasional giant cells. In some cases it was impossible to decide morphologically whether the disease present was granulomatous or neoplastic, and, in fact, a smooth transition in pathology appeared to be present in the mice, sometimes both diseases being present in the same animal.

Thus considerable uncertainty exists as to the true nature of the diseases developing in old age in the thymectomized mice, and before this situation can be clarified it may be necessary to carry out bacteriological, virological, and tissue transplantation studies on mice of this type.

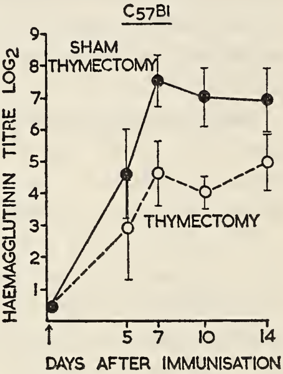
The present data provided no evidence that thymus exerts any humoral effect influencing the development of lymphoid leukemia. This conclusion is only tentative, for control experiments on the diffusion chambers of this type have revealed that thymus and spleen tissues survived very poorly in such chambers. After 1 to 2 months, it was rare to find significant amounts of viable tissue in the chambers, and it is most unlikely that any effective functional thymus tissue survived in the chambers used beyond the first few months after implantation. Until some superior form of diffusion chamber is developed, it cannot be asserted with certainty that a thymic humoral factor (which may need to be present for many months during leukemogenesis) is not involved in the development of lymphoid leukemia.

Immunological Responses in Adult Mice Following Thymectomy

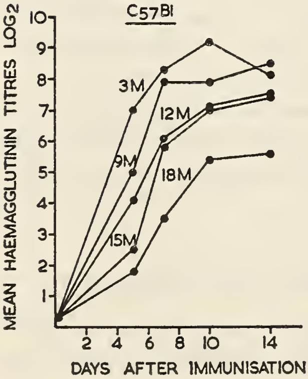
That many of the thymectomized mice in the preceding experiment appeared to be particularly susceptible to chronic infections raised the possibility that thymectomy in adult life might lead to the delayed development of depressed immune responses which could be of significance in the pathogenesis of various reticular tumors, *e.g.*, by allowing the emergence of antigenic tumor cells or by allowing chronic infections to provoke the development of reticulum cell sarcoma.

The effect of thymectomy on immune competence in adult mice was studied with sheep red cells as the antigen, since it has been demonstrated that immune competence to this antigen is depressed by neonatal thymectomy (*14*). Six-week-old C57BL and (AKR × C57BL)F_1 mice were thymectomized or sham-thymectomized, and tested at varying intervals after thymectomy. Mice tested 1 week after thymectomy produced a normal primary hemagglutinin response to sheep red cells. However, thymectomized mice of both strains exhibited depressed hemagglutinin responses when the mice were challenged for the first time 11 months after thymectomy (text-fig. 2). When (AKR × C57BL)F_1 mice were tested 18 months after operation (*18*), more than half the thymectomized mice were unable to produce detectable hemagglutinins.

It was also demonstrated that simple aging in mice of these two strains reduced immune responses to sheep red cells (text-fig. 3), and it seems likely that thymectomy in adult life accelerates the normal tendency for the development of depressed immune responses with advancing age.



Text-figure 2.—Primary hemagglutinin responses to sheep red cells in C57BL mice 11 months after thymectomy or sham thymectomy at the age of 6 weeks. Twelve mice in each group. *Vertical bars* indicate standard deviations.



Text-figure 3.—Primary hemagglutinin responses to sheep red cells in C57BL mice of various ages indicated in months (M). Each *point* represents mean value for 12 mice.

Kinetics of Polymorph and Monocyte Proliferation Following Thymectomy and Thymus Grafting

The possibility exists that the increased incidence of myeloid leukemia and reticulum cell sarcoma after thymectomy may result from the fact that the thymus may normally inhibit the proliferation of these cell types. On this hypothesis, thymectomy might then allow an increased proliferation of these cell types, which, if occurring in the presence of a viral agent capable of damaging these cells, might lead to the development of neoplasms of these cell types.

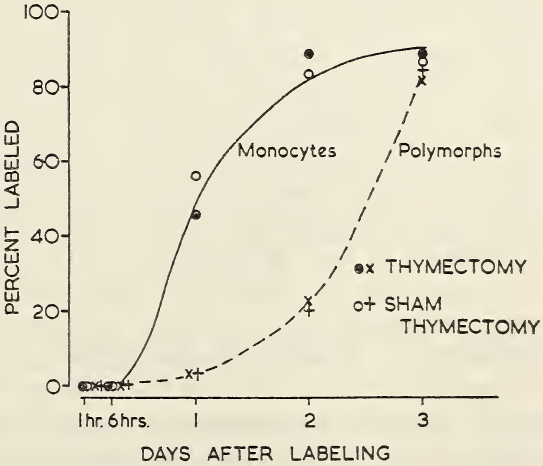
We have recently been carrying out autoradiographic studies on C57BL mice using tritiated thymidine labeling, and in the course of these experiments we have made some observations on the effect of thymectomy on 6-week-old mice on cellular proliferation in various tissues. As can be seen from text-figures 4 and 5, showing the rates of accumulation of labeled polymorphs and monocytes in the blood of thymectomized and sham-thymectomized C57BL mice, no difference was observed between the labeling patterns of normal C57BL mice and thymectomized C57BL mice when tested either 2 or 12 months after operation.

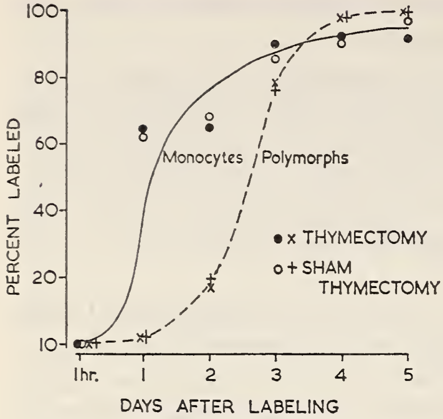
These data obviously are incomplete, but they do suggest that no obvious disturbances of cellular kinetics develop in the myeloid cell series or the monocytic cell series following thymectomy.

Similar negative effects of multiple thymus grafts were observed on polymorph and monocyte labeling patterns in C57BL mice carrying up to 48 C57BL thymus grafts each (*19*).

These studies obviously need to be reinforced by direct studies on the tissues of origin of these cell types (bone marrow and spleen) but, if the present indications are reliable, it seems most unlikely that the effect of thymectomy in inducing an increased incidence of myeloid leukemia and

TEXT-FIGURE 4.—Appearance of labeled polymorphs and monocytes in blood following tritiated thymidine labeling in C57BL mice 2 months after thymectomy or sham thymectomy at the age of 6 weeks. Each *point* represents mean value of 4 mice.





TEXT-FIGURE 5.—Appearance of labeled polymorphs and monocytes in blood following tritiated thymidine labeling in C57BL mice 12 months after thymectomy or sham thymectomy at the age of 6 weeks. Each *point* represents mean value of 4 mice.

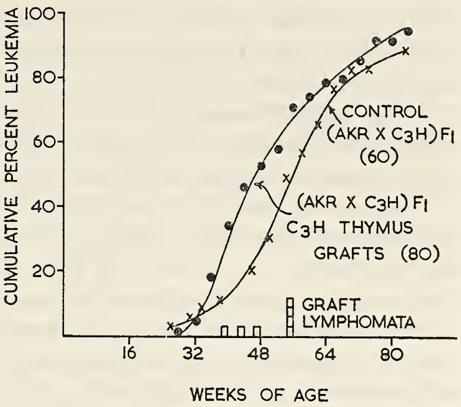
reticulum cell sarcoma is mediated via a disturbance in the population kinetics of these cell types.

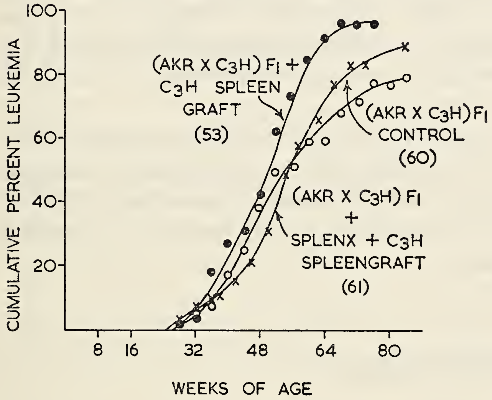
Effect of Thymus and Spleen Grafts From Low-Leukemia Parental Strains on Lymphoid Leukemia Development in F_1 Hybrid Mice

It might be postulated that the thymus in high-leukemia strain mice does not induce susceptibility to lymphoid leukemia by a positive influence, but rather that lymphoid leukemia develops because of a failure of the thymus in high-leukemia strain mice to exert some normal protective function in the body. Thus it was reported by Law (*20*) that when C3H thymus grafts were placed in thymectomized (C3H × AKR)F_1 hybrid mice no leukemia developed.

We have investigated this possibility by studying the effect of grafting two low-leukemia strain C3H neonatal thymus glands into young adult (AKR × C3H)F_1 mice and by grafting a C3H spleen into either intact (AKR × C3H)F_1 mice or into F_1 mice splenectomized immediately before grafting. These grafts were carried out when the recipient mice were 5 weeks old, and the grafted mice along with control mice were studied for the duration of their life for the incidence of lymphoid leukemia. The results obtained are shown diagrammatically in text-figures 6 and 7. It will be noted that neither C3H thymus grafts nor C3H spleen grafts had any significant inhibitory effect on the development of lymphoid leukemia in these F_1 hybrid mice. Of considerable interest was the observation that, in 7 of the 80 F_1 hybrid mice grafted with C3H thymus grafts, the lymphoma appeared to originate in the low-leukemia strain thymus graft. In such mice the thymus graft was replaced by a massive lymphosarcoma, while the host thymus was either normal or only slightly infiltrated by lymphoma cells and there was a variable degree of involvement of other host lymphoid organs by leukemic cells.

TEXT-FIGURE 6.—Cumulative mortality from lymphoid leukemia in control (AKR × C3H)F_1 mice and (AKR × C3H)F_1 mice grafted subcutaneously with 2 C3H thymus grafts. Number of mice in each group is indicated in parentheses. *Blocks* indicate the occurrence of primary lymphomas in the C3H thymus grafts.





TEXT-FIGURE 7.—Cumulative mortality from lymphoid leukemia in control (AKR × C3H)F_1 mice and splenectomized and sham-operated (AKR × C3H)F_1 mice grafted subcutaneously with single C3H spleen grafts. Number of mice in each group is indicated in parentheses.

These experiments do not support the concept that a high-leukemia strain thymus exerts a leukemogenic effect by default—that is, by being unable to perform a normal protective function usually carried out by the thymus in other strains. Thymus grafts are known to be repopulated by cells of host origin (*21*), and it is evident from the results obtained with F_1 hybrid mice grafted with C3H thymus that the repopulating cells in some of the grafts developed neoplastic change despite the fact that they were resident within the epithelial and reticulum cell meshwork of a low-leukemia strain thymus. This result would suggest that the primary factor affecting lymphoma development in a thymus is the inherent susceptibility of the lymphoid cells of the thymus to neoplastic change (in the presence of leukemia virus) and not the functional state of the epithelial and reticulum cell elements of the thymus.

Effect of Multiple Thymus Grafting on Development of Lymphoid Leukemia

It has been postulated that the susceptibility of the thymus to lymphoma development stems from the extensive lymphopoiesis occurring in this organ and that neoplastic cells may arise by random mutations, the frequency of such neoplastic mutations depending on the frequency of mitosis. If this hypothesis is correct, then it should be possible, by placing many thymus grafts in the same recipient animal, to increase the risk of development of lymphoid leukemia in that animal simply by creating massive amounts of thymic tissue exhibiting intense lymphopoiesis.

We have studied the effect of grafting 10 neonatal C3H thymus grafts to C3H recipient mice. These mice were grafted initially when 4 weeks old by placing 2 neonatal C3H thymus grafts in the subcutaneous tissues. At 2 monthly intervals thereafter, an additional 2 neonatal thymus grafts were placed in the subcutaneous tissues, until a total of 10 thymus grafts had been placed in each recipient animal. By this technique, it was hoped to have a continuous supply of rapidly proliferating thymus tissue in the animal for the first 8 months of life. Thirty-five mice given multiple thymus grafts were studied for life for the development of lymphoid leukemia, but these mice did not exhibit any higher incidence of lymphoid leukemia than control C3H mice (5%).

In other experiments we have grafted from 12 to 48 thymus grafts from C57BL mice to C57BL recipients, and although no specific study was made of lymphoid leukemia development in these mice, in more than 100 such mice that were observed for other reasons for up to 12 months, no apparent lymphoid leukemia has developed.

DISCUSSION

The present results have confirmed that thymectomy in adult life reduces the incidence of lymphoid leukemia and prolongs the latent period to lymphoid leukemia development of those few mice developing lymphoid leukemia. The present results have also confirmed that such thymectomized mice have an increased incidence of both myeloid leukemia and reticulum cell sarcoma in later life. It has been postulated that the increased incidence of myeloid leukemia and reticulum cell sarcoma following thymectomy stems simply from the fact that such mice live longer than unoperated mice normally do and are merely expressing susceptibility to disease development which normally would have been expressed had they lived long enough. The present experiments support such an interpretation, although they do not prove it.

Although there are some reservations regarding the efficiency of the diffusion chambers used in our experiments, the present results provide no indication that a humoral factor produced by the thymus is of significance in the development of spontaneous lymphoid leukemia in thymectomized high-leukemia strain mice. However, in earlier experiments with high-leukemia strain thymus grafts in low-leukemia F_1 hybrid mice, evidence was obtained that the thymus might act at a distance to induce an increased incidence of leukemia development, suggestive of the involvement of a humoral factor (*22*). This question cannot really be finalized until an improved diffusion chamber is developed which can guarantee survival and functional activity of thymus tissue for prolonged periods.

The present experiments have demonstrated that thymectomy in adult life is followed by the delayed development of a significant depression of immune competence. The age at which myeloid leukemia and reticulum cell sarcoma, or for that matter the delayed lymphoid leukemia, develop in adult thymectomized mice is the same age at which it is possible to demonstrate such depressed immune responses in adult thymectomized mice. It is possible, therefore, that at least some of the reticular tumors developing in thymectomized mice may do so because of depressed or inadequate immune responses. Obviously this question can only be settled by the direct demonstration that the tumor cells in such diseases do contain antigens foreign to the host and that thymectomized mice in middle and old age are less competent to reject test transplants of such tumor cells than are normal mice of the same age.

No evidence was obtained from studies with tritiated thymidine that thymectomy affects the proliferative rate of myeloid or monocytic cells, and it seems unlikely that the effect of thymectomy in allowing an increased incidence of tumors of these cell types is directly attributable to modifications in the proliferative activity or maturation of these cell types.

No evidence was obtained from the present studies that the thymus in high-leukemia strain mice exerts its leukemogenic influence by a failure to perform normal functions. The present data support earlier conclusions that the leukemogenic influence of a high-leukemia strain thymus is a positive, rather than a negative, influence.

The present interest in the thymus as an organ regulating immune responses and the recent evidence that lymphoid leukemic cells are antigenic for the host animal raise the possibility that the thymus may influence lymphoid leukemia development by modifying some aspect of host immune responses to such emerging antigenic tumor cells.

SUMMARY

Thymectomized (AKR × C3H)F_1 hybrid mice had a reduced incidence of lymphoid leukemia but an increased incidence late in life of myeloid leukemia, reticulum cell sarcomas, and deaths from chronic infections. AKR thymus or spleen grafts in diffusion chambers had no significant influence, either on the incidence of lymphoid leukemia or on the incidence of myeloid leukemia and reticulum cell sarcoma development. Thymectomy had no effect on blood polymorph or monocyte population kinetics.

Thymectomy in adult life was found to lead to the delayed development of a depressed immune response to the primary injection of sheep red cells, and part of the role of the thymus in leukemia development may be due to

its influence on immune responsiveness, since virus-induced leukemic cells are antigenic.

No evidence was obtained that low-leukemia strain thymus or spleen grafts in intact F_1 hybrid mice protected these mice from lymphoid leukemia development, and in some cases lymphomas appeared to arise in the low-leukemia strain C3H thymus grafts in such mice.

REFERENCES

(*1*) McEndy, D. P., Boon, M. C., and Furth, J.: On the role of the thymus, spleen, and gonads in the development of leukemia in a high-leukemia stock of mice. Cancer Res 4: 377–383, 1944.

(*2*) Gross, L.: "Spontaneous" leukemia developing in C3H mice following inoculation, in infancy, with AK-leukemic extracts, or AK-embryos. Proc Soc Exp Biol Med 76: 27–32, 1951.

(*3*) ———: Pathogenic potency and host range of the mouse leukemia virus. Acta Haemat (Basel) 29: 1–15, 1963.

(*4*) ———: Effect of thymectomy on development of leukemia in C3H mice inoculated with leukemic "passage" virus. Proc Soc Exp Biol Med 100: 325–328, 1959.

(*5*) Levinthal, J. D., Buffett, R. F., and Furth, J.: Prevention of viral lymphoid leukemia in mice by thymectomy. Proc Soc Exp Biol Med 100: 610–614, 1959.

(*6*) Miller, J. F. A. P.: Role of the thymus in murine leukaemia. Nature (London) 183: 1069, 1959.

(*7*) ———: Recovery of leukaemogenic agent from nonleukaemic tissues of thymectomized mice. Nature (London) 187: 703, 1960.

(*8*) ———: Role of the thymus in virus-induced leukaemia. *In* Ciba Symposium on Tumour Viruses of Murine Origin (Wolstenholme, G. E. W., ed.). London, Churchill, 1962, pp 262–279.

(*9*) Gross, L.: Development of myeloid (chloro-) leukemia in thymectomized C3H mice following inoculation of lymphatic leukemia virus. Proc Soc Exp Biol Med 103: 509–514, 1960.

(*10*) Kunii, A., and Furth, J.: Inhibition of lymphoma induction in virus-infected rats by thymectomy. An affinity of the lymphoma virus for myeloid cells. Cancer Res 24: 493–497, 1964.

(*11*) Metcalf, D.: The thymic origin of the plasma lymphocytosis stimulating factor. Brit J Cancer 10: 442–457, 1956.

(*12*) ———: The thymic lymphocytosis stimulating factor and its relation to lymphoid leukemia. *In* Canadian Cancer Conference III (Begg, R. W., ed.). New York, Academic Press Inc., 1959, pp 351–366.

(*13*) ———: Longterm effects of whole body irradiation on lymphocyte homeostasis in the mouse. Radiat Res 10: 313–322, 1959.

(*14*) Miller, J. F. A. P.: The thymus and the development of immunologic responsiveness. Science 144: 1544–1551, 1964.

(*15*) Slettenmark, B., and Klein, E.: Cytotoxic and neutralization tests with serum and lymph node cells of isologous mice with induced resistance against Gross lymphomas. Cancer Res 22: 947–954, 1962.

(*16*) Dunn, T. B.: Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J Nat Cancer Inst 14: 1281–1433, 1954.

(*17*) Metcalf, D., and Wiadrowski, M.: Autoradiographic analysis of lymphocyte proliferation in the thymus and in thymic lymphoma tissue. Cancer Res. In press.

(*18*) METCALF, D.: Delayed effect of thymectomy in adult life on immunological competence. Nature (London). In press.

(*19*) MATSUYAMA, M., WIADROWSKI, M., and METCALF, D.: Unpublished data.

(*20*) LAW, L. W.: Some aspects of the etiology of leukemia. *In* Canadian Cancer Conference III (Begg, R. W., ed.). New York, Academic Press Inc., 1959, pp 145–169.

(*21*) METCALF, D., and WAKONIG-VAARTAJA, R.: Stem cell replacement in normal thymus grafts. Proc Soc Exp Biol Med 115: 731–735, 1964.

(*22*) METCALF, D.: Thymus grafts and leukemogenesis. Cancer Res 24: 1952–1957, 1964.

DISCUSSION

Dr. Dunn: I certainly followed Dr. Metcalf's work with great interest, and he is making a very important contribution to our understanding of the effect of thymectomy on leukemia development. My own experience in thymectomized mice and the development or the changing pattern of leukemia occurred with BALB/c mice infected with the Moloney virus. We found that among the thymectomized mice—I think there were 20 to begin with—only 1 developed lymphocytic leukemia. However, the thymectomized mice lived much longer than intact mice infected with the virus, and we found that many developed reticulum cell tumors and also a few myeloid leukemias.

I have presumed from this that the removal of the most susceptible organ allowed the opportunity for the development of other tumors at a later date. In fact, we thought the situation was somewhat parallel to our experience with C3H mice in which the milk agent is removed. In these mice we failed to get the typical milk agent tumors at an early age but in the longer surviving mice we got a variety of morphological types of mammary tumor.

My other wish is that more work was being done with the C58 mouse. We are told the thymus is unimportant in this strain, although certainly some of Dr. Law's work is contrary to this opinion. But so far as I could tell in all of Richter's papers I reviewed on his observations on the C58, he never once mentioned the thymus. Also, the typical preleukemic lesion described in this strain was a change in the lymph nodes.

Dr. Metcalf: I did not emphasize it, but the thymectomized mice lived longer than the unoperated mice. The usual explanation for the usual tumors developing in thymectomized mice is that all mice will show this spectrum of tumor development if only they can live long enough. Even in the sham-operated mice there were a few cases of reticulum cell tumor.

Your point about the C58 mice is well taken. This is the exception to most mouse strains in that lymphoid leukemia in C58 mice does not appear to arise in the thymus, although thymectomy prevents the disease.

All I can say, Dr. Dunn, is that you must never have tried to breed C58 mice. They are extremely difficult to breed and this is the reason why few people work with them.

Dr. Dunn: Dr. Law has some that I've had an opportunity to look at, and I hope we can follow them a little more closely.

Chairman Furth: There is a tendency not to attribute every neoplasm that occurs in old thymectomized mice to the virus. Old animals are susceptible to many spontaneous neoplasms. How are you going to sort out what is virus-induced and what is not? Do you have the answer, Don?

Dr. Metcalf: If these tumors contain the G antigen it may indicate that the Gross virus is etiologically involved. It wouldn't prove it, but it's suggestive—at least in mice like the (AKR × C57BL)F_1 when G antigen is either absent or only present in small amounts.

Chairman Furth: I don't think you'll get very far because the G antigen is merely a viral antigen present in AKR mice before and after the onset of leukemia, present as long as the virus is there.

Dr. Metcalf: It may be better to use a viral induction system to try to induce viral release by irradiating the tumor cells.

Dr. Kirsten: I would like to make a brief comment on Dr. Metcalf's experiment with the diffusion chamber. Dr. Hefter in our laboratory has been trying to study leukemogenesis *in vitro* using Dr. Auerbach's model of organ culture of embryonic thymus. Whereas lymphoid differentiation occurs in the normal, uninfected organ culture, Gross virus in the culture supernatant either prevents or retards lymphoid differentiation in embryonic thymic organ cultures. One ends up with a thymus depleted of lymphoid cells; instead, it is composed of the epithelial reticular element.

This seems to suggest there are other factors needed, at least *in vitro*, to produce a leukemic thymus. Several technical difficulties, namely, the continued growth of the thymus which finally outgrows its nutrient supply, make it impossible to study this system for a long time, but one has the impression that there might be a humoral factor lacking in this artificial system.

Dr. Fiore-Donati: The negative results on the influence of thymus on bone marrow were obtained by Dr. Metcalf, I think, in thymectomized adult mice. On the other hand, morphologic signs of proliferation and arrest of maturation of myeloid tissue have been observed in neonatally thymectomized animals by Miller *et al.* (Proc. Soc. Exp. Biol. Med., 118: 916–921, 1965), by Parrott and East [Nature (London) 195: 347–348, 1962], and by Law *et al.* [*In* The Thymus (Defandi, V., and Metcalf, D., eds.). Philadelphia, The Wistar Institute Press, 1964, pp. 105–117]. Thus, it may well be that the age at which thymectomy is performed is important in regard to the possibility of detecting a direct effect of thymus on the bone marrow.

Dr. Metcalf: I believe other groups have attempted to restore leukemia susceptibility with thymus grafts in diffusion chambers in mice subjected to neonatal thymectomy (in our case it was adult thymectomy) and injected with passaged leukemia virus. But as far as I know all these attempts have been failures.

Dr. Chieco-Bianchi: Has anybody tried to restore immunological competence of neonatally thymectomized mice by lymphomatous thymic tissue in diffusion chambers or by cell-free extracts of leukemic tissue of thymic origin?

Dr. Metcalf: Neonatally thymectomized mice were not restored to normal by injections of thymus extract [Miller, J.F.A.P. *In* The Thymus in Immunobiology (Good, R. A., and Gabrielson, A. E., eds.). New York, Harper and Row, 1964, p. 436]. Large doses of thymus cells are effective but not as effective as adult spleen cells, and, of course, thymus grafts in diffusion chambers are effective.

Then procedures result in only a temporary cure of the neonatal thymectomized animal. If one follows them long enough such mice will ultimately again deteriorate and die prematurely (Law, personal communication). But, for a time, cell suspensions from adult animals are effective in restoring a normal hematological picture and immune competence.

Dr. Chieco-Bianchi: Do you agree on this assumption that thymus is as essential for lymphoid neoplastic transformation as it is for development of immune competence of normal lymphoid cells in physiological conditions?

Dr. Metcalf: As you know, the AKR mouse is specifically tolerant of the Gross (G) antigen (Axelrad, A., Prog. Exp. Tumor Res. 6: 30, 1965), and at first sight it might appear that the questions of immune competence do not arise during lymphoid leukemia development. However, we've been struck by the close similarity between the role of the thymus in immune tolerance and the relation of the thymus to leukemia development.

In brief, in a tolerant animal, immune tolerance tends to break down in old age. Thymectomy delays or prevents this breakdown in tolerance. Irradiation accelerates the breakdown of tolerance. Similarly, thymectomy delays or prevents lymphoid leukemia development and whole-body irradiation is leukemogenic. We wonder whether in the late preleukemic period in the tolerant animal there may not be a local breakdown in immune tolerance to the G antigen in the thymus. In the medulla of the AKR thymus late in the preleukemic period, one sees lymphoid follicles and germinal centers developing which are the classic morphological expressions of a local immune response. At this point, it is purely speculative, but we wonder whether this expression of an immune response indicates a local response to the G antigen and whether this is related in some way to subsequent neoplastic change in this tissue.

Dr. Block: There has evidently been some question raised about the reciprocal relationship between development of lymphoid neoplasms and of granulocytic neoplasms. It was pointed out that in the thymectomized mouse there was a higher incidence of subsequent development of myeloid—we prefer to call it granulocytic-type neoplasms. I have some observations made on an entirely different animal which bear on this.

When the neonatal opossum is thymectomized (and we pick the opossum because at birth its thymus lacks lymphatic tissue and the first lymphatic tissue appears in cervical lymph nodes at 3 days, in the mediastinal lymph nodes at 5 days, and in the spleen at 18 days), we could show that there was indeed a reciprocal relationship between myeloid and lymphatic tissue. Thymectomy, prior to development of lymphatic tissue in the spleen, not only inhibits development of this lymphatic tissue in the spleen but permits unrestrained growth of myeloid tissue in the spleen. The spleens become huge and filled with erythroblasts, granulocyte precursors, and megakaryocytes. This is anticipated because the spleen is embryologically and phylogenetically not a lymphatic but a myeloid organ. The "embryonic" thymus evidently suppresses the myeloid tissue and this suppression then allows the lymphatic tissue to develop.

Thymectomy had no effect on the development of bone marrow. Bone marrow development was similar in thymectomized and in nonthymectomized animals. In all cases, the thymectomies were done before the development of bone marrow.

Dr. Gross: We have been breeding C58 mice for a number of years in our laboratory and had no difficulty breeding them by brother × sister mating. The incidence of thymic tumors observed in spontaneous C58 leukemia was about 85% in our small colony.

Dr. Klein: With regard to your experiment concerning the possible surveillance function of the low-leukemic thymus, I wonder if you have done a similar experiment with a hybrid like (AKR × C57BL) F_1 thymus and C57BL thymus?

Dr. Metcalf: No, we haven't. Actually this surveillance function is most unlikely because, as Stich and his group have shown (Joreja, M. G., and Stich, H. F., Exp. Cell. Res. 31:220, 1963), the thymus is the site par excellence for the accumulation and proliferation of abnormal cells during the preleukemic period, at least as judged by cells with abnormal chromosomes.

I don't really believe that low-leukemia strain thymus grafts would protect against leukemia development, but it is perhaps worth checking another strain combination.

The comment by Dr. Block is well taken. Our failure to find evidence that the thymus influences myelopoiesis in the adult mouse may be a little misleading, and it may not be true in neonatal mice or in other species. For example, we have put neonatal spleen grafts into adult thymectomized animals and show normal lymphoid development and normal myelopoiesis in the spleen grafts. But Good (personal communication) has done the same type of experiment using neonatally thymectomized recipient mice and has evidence that myelopoiesis was excessive in these grafts in the young hosts. It may be that the thymus influences myelopoiesis in early life but not in adult life.

Studies on Thymus-Dependent Mechanisms of Mouse Leukemogenesis by Graffi Virus [1, 2]

Luciano Fiore-Donati, Luigi Chieco-Bianchi, Giuseppe Tridente,[3] *and* Natale Pennelli, *Istituto di Anatomia e Istologia Patologica, Divisione di Cancerologia Sperimentale, Università di Padova, Padova, Italy*

In previous experiments on the leukemogenic potentiality of Graffi virus in mice, the relative incidence of different types of leukemia induced by this virus varied greatly according to the environmental conditions of the host (*1*). Some of the strains tested, all of which proved highly susceptible to the virus, developed myeloid leukemia in a high percentage. In other strains, such as C3Hf/Gs and AKR, lymphatic leukemia occurred almost exclusively. Thymectomy performed at 30 days of age in C3Hf/Gs and AKR mice caused a striking change in the morphologic type of leukemic response, significantly increasing the ratio of myeloid to lymphatic forms. However, the total incidence of leukemia remained practically unchanged.

Furthermore, morphologic studies showed that in nonthymectomized C57BL mice developing myeloid leukemia the thymus appeared not involved by the disease or exhibited, in most cases, neoplastic transformation only of lymphatic type (*1, 2*).

The present investigations were undertaken to elucidate the thymus-dependent mechanisms by which Graffi virus induces a selective transformation of cells of either lymphoid or myeloid tissues. This paper describes the results of experiments concerning: (*a*) an attempt to restore the susceptibility to lymphatic leukemia induction, depressed by thymectomy, by grafting thymus in mice inoculated with virus; (*b*) the possible influence of different time intervals between virus inoculation and thymectomy, as well as between thymectomy and thymus reimplantation, on the morphology of virus-induced leukemia; (*c*) the morphologic modifi-

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported in part by grant DRG-808 from the Damon Runyon Memorial Fund for Cancer Research and by a grant from Consiglio Nazionale delle Ricerche, Roma.

[3] Fellow of Lady Tata Memorial Fund.

cations of thymus grafts in virus-inoculated, thymectomized mice, reimplanted with newborn thymus from normal parental donors.

MATERIALS AND METHODS

($C3Hf/Gs \times CBA/H)F_1$ hybrid mice of both sexes were used throughout the experiments. The parental strains were both obtained from the Chester Beatty Research Institute, London, through the courtesy of Dr. J. F. A. P. Miller. The hybrids received subcutaneously, at the age of 12 to 48 hours, 0.05 ml of cell-free extract containing Graffi virus. The extracts obtained from C57BL mice with generalized leukemia induced by Graffi virus were prepared as previously reported (*1*). The animals were weaned at the age of 5 to 6 weeks and separated according to the sex. They were kept in wooden cages, 5 to 6 per cage, in temperature-controlled rooms at 21 to 23° C, and fed on ARSAL mouse diet and water *ad libitum.*

To investigate the thymus-dependency of the morphology in viral leukemogenesis, the following experiments were undertaken. In one experiment, groups of mice were thymectomized at various intervals from virus inoculation, *i.e.*, at the age of 3, 20, 30, 40, or 60 days. An additional group consisted of mice submitted to removal of only the right lobe of thymus, at 30 days of age. In another experiment, groups of virus-injected mice, all thymectomized at 30 days of age, were grafted at 7, 20, or 40 days after thymectomy with a single newborn thymus from normal C3Hf/Gs donors. In an additional group, 5 thymuses instead of a single one were implanted 7 days after thymectomy. A small group of mice thymectomized at 30 days of age received intraperitoneally, 7 days after thymectomy, Millipore diffusion chambers (pore size 0.45 μ) containing one lobe of newborn C3Hf/Gs normal thymus. Control mice were implanted with empty chambers. Diffusion chambers were prepared according to the technique of Osoba and Miller (*3*).

Thymectomy and thymus grafting were performed under ether anesthesia; the grafts were implanted subcutaneously into the right flank.

Animals on which no surgery was performed were inoculated at birth with virus preparations, as the experimental animals, and served as controls.

Mice were examined twice weekly for the development of leukemia and gross modifications of thymus grafts. Usually they were killed when moribund, and all organs, except the brain, were examined for leukemic infiltration. Special care was taken at autopsy to ascertain the completeness of thymectomy, the presence of thymus grafts, and their gross characteristics. Among thymectomized mice, only those showing no residual thymus *in situ* on histologic examination were included in the study. In the groups implanted subcutaneously with thymus(es), animals in which the grafts were not recovered at autopsy were discarded.

The following organs and tissues were routinely examined histologically: inguinal, mesenteric, and submaxillary lymph nodes, fragments of liver, spleen, and lungs, and thymus with mediastinal tissues. Occasionally kidneys, adrenal glands, ovaries, and segments of spinal column with perivertebral tissues were also taken. In thymectomized mice the upper mediastinum was removed en bloc. In thymus-implanted mice the grafts were removed and examined with the surrounding tissues. Sections were fixed in Bouin's fluid and stained with hematoxylin and eosin. Blood smears, performed immediately before the animals were killed, were stained with May-Grünwald-Giemsa.

RESULTS

The total incidence and latency of leukemias in virus-inoculated hybrid mice thymectomized at various ages, and in mice thymectomized at 30 days and then receiving implants of thymus at intervals after thymectomy, are shown in tables 1 and 2.

It is evident that the removal of thymus did not influence the susceptibility of mice to leukemogenic activity of the virus. The total incidence of leukemia in thymectomized mice, whether reimplanted with thymus or not, was practically comparable to that of intact animals. The median total latency, however, was remarkably prolonged, from 18 weeks in unoperated controls to 25 to 34 weeks in thymectomized mice. On the other hand, mice receiving thymus grafts developed leukemia after a median latent period of 20.5 to 25 weeks, which was intermediate between those of intact and thymectomized animals.

The effect of thymectomy on the type of leukemic response induced by the virus is shown in table 3. It can be seen that removal of the thymus caused a remarkable modification of relative incidences of the different types of leukemia. Myeloid leukemias, often exhibiting the aspect of chloroleukemia, developed in a considerable number of thymectomized mice, with an incidence ranging from 100 percent in animals thymecto-

TABLE 1.—Total incidence and latency of leukemias developing in (C3Hf/Gs × CBA/H)F_1 hybrid mice inoculated at birth with Graffi virus and thymectomized at various ages

Age at thymectomy (days)	Number of leukemic mice / Total No. of mice	Incidence* (%)	Latency (weeks) † (median)
3	21/25	84	25
20	22/30	73	29
30	35/38	92	29
40	32/34	94	34
60	17/21	81	26
—	54/57	95	18

*Percent based on number of mice living at time of first leukemia

†Corresponding to age of animals at death.

TABLE 2.—Total incidence and latency of leukemias developing in (C3Hf/Gs × CBA/H)F_1 hybrid mice inoculated at birth with Graffi virus, thymectomized at 30 days of age, and grafted subcutaneously at different ages with single or multiple newborn thymuses from normal C3Hf/Gs

Number of thymus grafts	Time of grafting after thymectomy (days)	Number of leukemic mice / Total No. of mice	Incidence* (%)	Latency (weeks)† (median)
1	7	35/35	100	25
1	20	20/24	83	20. 5
1	40	20/25	80	24
5	7	22/22	100	20. 5
—	—	35/38	92	29

*Percent based on number of mice living at time of first leukemia.

†Corresponding to age of animals at death.

mized at 3 days to 53 percent in animals thymectomized at 60 days. In mice thymectomized at 3 days, however, myeloid leukemia appeared after a latent period much shorter than in mice of other groups and in only 1 of the 21 cases showed the characteristic green of chloroleukemia. No evidence of wasting was noted in these animals.

In the group of 24 mice partially thymectomized, not included in the table, lymphatic leukemia occurred in 14 (58%), myeloid leukemia in 8 (33%), and reticulum cell sarcoma in the remaining 2 animals (8%).

In controls without surgery, the incidence of lymphatic leukemia was 88 percent, while myeloid forms occurred in only 10 percent and other leukemic forms in the remaining 2 percent.

In regard to the influence of time at which thymectomy was performed, no significance differences were noted among the various groups thymectomized between 3 and 40 days after virus inoculation. On the other hand, in mice thymectomized at 60 days the incidence of myeloid leukemia was remarkably lower (53%) and that of lymphatic leukemia higher (29%) than in the other groups.

Table 4 provides data on the restoring effect of thymus grafting on the susceptibility to lymphatic leukemia induction depressed by thymectomy. In the various groups of thymectomized animals grafted with single or multiple thymuses at different intervals after thymectomy, the incidence of lymphatic leukemia was significantly higher than in thymectomized control mice, although it remained somewhat below the incidence observed in intact mice. No significant variations were observed in the ratio of lymphatic to myeloid forms in relation to the different time intervals between thymectomy and thymus reimplantation. However, in mice grafted with 5 thymuses the incidence of lymphatic leukemia was higher (71%) and that of myeloid leukemia lower (24%) than in mice grafted with a single thymus at the same interval from thymectomy (51 and 43%, respectively).

Implantation of thymic tissue in diffusion chambers in mice thymectomized at 30 days of age failed to restore susceptibility to lymphatic leu-

TABLE 3.—Incidence and latency of different types of leukemia developing in (C3Hf/Gs×CBA/H)F_1 hybrid mice inoculated at birth with Graffi virus and thymectomized at various ages

		Type of leukemia					
		Lymphatic		Myeloid		Others*	
Age at thymectomy (days)	Number of leukemic mice examined	Incidence† (%)	Latency (weeks)‡ (median)	Incidence† (%)	Latency (weeks)‡ (median)	Incidence† (%)	Latency (weeks)‡ (median)
3	21	0	—	100	25	0	—
20	22	9	24	82	30. 5	9	27, 44
30	34	3	15	85	31	12	23. 5
40	28	14	30	82	34	4	25
60	17	29	16	53	30	18	24
—	41	88	17	10	27	2	24

*Including: stem cell, reticular, and unclassified types.

†Percent based on number of mice living at time of first leukemia.

‡Corresponding to age of animals at death.

Table 4.—Incidence and latency of different types of leukemia developing in (C3Hf/Gs × CBA/H)F_1 hybrid mice inoculated at birth with Graffi virus, thymectomized at 30 days of age, and grafted subcutaneously at different ages with single or multiple newborn thymuses from normal C3Hf/Gs mice

Number of thymus grafts	Time of grafting after thymectomy (days)	Number of leukemic mice examined	Type of leukemia					
			Lymphatic		Myeloid		Others*	
			Incidence† (percent)	Latency (weeks)‡ (median)	Incidence† (percent)	Latency (weeks)‡ (median)	Incidence† (percent)	Latency (weeks)‡ (median)
1	7	35	51	22	43	32	6	16
1	20	22	64	20	27	20	9	23
1	40	20	60	23	35	26	5	24
5	7	21	71	20	24	22	5	24
—	—	34	3	15	85	31	12	23. 5

*Including: stem cell, reticular, and unclassified types.

†Percent based on number of mice living at time of first leukemia.

‡Corresponding to age of animals at death.

kemia induction. Lymphatic leukemia was not observed in any of the 12 leukemic mice of this group. Ten mice developed myeloid leukemia, while the other 2 animals had reticulum cell sarcoma. Comparable results were obtained in the 9 thymectomized control mice bearing empty diffusion chambers (7 myeloid leukemias, 1 reticulum cell sarcoma, 1 leukemia of unclassified type).

Morphology

Lymphatic and myeloid leukemias showed anatomical and hematological features comparable to those previously reported in mice of several strains inoculated with Graffi virus (*1*). The lymphatic leukemias were of a generalized type, with massive enlargement of thymus. Histologically, the affected organs were infiltrated by uniform sheets of neoplastic cells showing the morphological characteristics of immature lymphoid cells (lymphoblasts). Mice with myeloid leukemia showed enlargement of lymph nodes, spleen, and liver. In the few intact mice with this type of leukemia, the thymus appeared either normal or only slightly enlarged, and it was clearly distinguishable by its pearly white color from the mediastinal greenish lymph nodes. Histologically, the thymic tissue in these mice was atrophic or replaced by neoplastic cells of lymphoid type, thus differing from myeloid leukemic cells infiltrating the other organs, *i.e.*, lymph nodes, spleen, and liver. Myeloid neoplastic tissue was composed of cells of the granulocytic series in all stages of differentiation, though the prevailing cellular type varied greatly in different cases.

In the thymectomized mice leukemic disease of both types was substantially similar to that of intact mice. However, 14 of the 122 thymectomized mice examined showed signs of paralysis of hind limbs. In these animals myeloid infiltration of the lumbar part of the spinal column was observed histologically. Similar findings were previously reported by us in other strains of thymectomized mice injected with Graffi virus (*1*) and by Levy *et al.* in rats injected with Gross virus (*4*).

In most animals of the various groups, immature leukemic cells of either lymphatic or myeloid series were detected in the peripheral blood.

The modifications of implanted thymuses were found to differ according to the type of leukemia occurring in the host. In animals developing lymphatic leukemia the neoplastic disease seemed to initiate as a localized growth at the graft site, rapidly reaching a remarkable size and then followed by generalization of the leukemic process. Histologically, the thymus graft was entirely composed of lymphoid neoplastic cells with morphologic characteristics identical to those of leukemic cells in the other organs affected. Sometimes, however, islands of medullary tissue were easily recognizable (fig. 1). On the other hand, when myeloid leukemia developed in spite of thymus inplantation, the graft occasionally retained the original size and showed the architectural pattern of the normal organ.

Usually, it increased slowly to a moderate size and appeared involved by the leukemic process. In all these cases, however, the type of neoplastic cells replacing thymic tissue was clearly lymphoid.

It is noteworthy that some of these grafts were partially surrounded and/or infiltrated by myeloid host cells at the periphery (figs. 2, 3, and 4). The coexistence of two different neoplastic tissues was further proved by transplantation assay. Fragments of lymphoid neoplastic thymus grafts and myeloid nodes obtained from the same leukemic animal were successfully transplanted to syngeneic mice, which led to development of both lymphoid and myeloid local tumor growths.

DISCUSSION

The present experiments concern the functional and morphologic behavior of thymus in leukemia induced by Graffi virus in mice.

As indicated by previous observations, the anatomical expressions of leukemic response to this virus appear to be mainly conditioned by factors contributed by the recipient host. The data presented here suggest that the capability of Graffi virus to induce neoplastic transformation of one type or another of hematopoietic cells depends to a considerable extent on whether the thymus is present.

In the hybrids used in these experiments, as well as in other strains previously tested, lymphatic leukemia developing in a high percentage of intact mice was prevented almost completely by removal of the thymus as late as 30 to 40 days after virus inoculation.

Thymus grafts restored, at least partially, the susceptibility to lymphatic leukemia induction by the virus. The highest incidence of lymphatic leukemia was observed in mice grafted with multiple thymuses. This finding parallels the results obtained in another series of experiments in which a chemical agent, such as urethan, induced in adult C57BL mice a significant number of malignant lymphomas only when the treated animals were implanted with multiple instead of single syngeneic thymus grafts (*5*).

In all thymectomized, thymus-implanted mice where the host had lymphatic leukemia, the graft appeared to be the primary site of origin of the neoplastic disease. In these mice the grafted thymus was the organ most severely affected and the first to undergo neoplastic changes. It follows from all these data that also in leukemogenesis by Graffi virus, the thymus, either *in situ* or grafted in thymectomized host, is essential for lymphatic leukemia induction.

These results are in accordance with those obtained by others in different host-virus systems (*6–14*) or in radiation-induced leukemia (*15*, *16*), showing that thymectomy provokes a striking change in the relative percentages of different types of leukemia, being followed by neoplastic transformation of hematopoietic cells other than lymphatic. In our experiments, a considerable shift of malignant changes from lymphoid to myeloid tissues was also observed in mice submitted to partial thymectomy. However, in 6 of 8 mice developing myeloid leukemia no residual thymic fragments could be recovered at autopsy. Thus, it remains uncertain whether myeloid leukemia in this group had occurred simply as a result of complete absence of thymus due to atrophy of the unremoved thymic lobe.

As judged from this and similar studies of others, it would appear that induction of myeloid leukemia by viruses or X irradiation is not thymus-dependent, at least in the sense of a direct influence of thymic tissue on myeloid cells. Therefore one may assume, as first suggested by Furth (*6*), that if an animal is spared from lymphatic leukemia by removal of the lymphoid (thymic) cells most susceptible to neoplastic transformation, other late-occurring neoplasms of reticular system may eventually arise. However, recent reports suggest the thymus also has a function in controlling the maturation and proliferation of myeloid tissue. In a study on the effect of thymectomy on hematopoietic organs of the opossum "embryo," Miller *et al.* (*17*) found a remarkable increase of myeloid tissue, mainly in spleen and lymph nodes, associated with abnormal proliferation and arrest of maturation. This and other similar observations in mice (*18*, *19*) raise the possibility that thymectomy exerts a favoring effect on myeloid leukemia induction, not only through elimination of cells preferentially sensitive to the action of certain leukemogenic agents but also by abolishing the inhibitory influence of the organ on myeloid tissue. If so, myeloid leukemia also could be considered, broadly speaking, a disease conditioned by thymus-dependent mechanisms.

The experiments dealing with the effect of thymectomy at different intervals showed that removal of thymus as late as 40 to 60 days after virus inoculation was less effective in preventing lymphatic leukemia than if performed earlier. This would suggest that at this time a certain number of neoplastic cells of thymic origin may have already been seeded in peripheral lymphoid tissues or, alternatively, that extra-thymic lymphoid cells have already been transformed under the functional activity of the thymus. On the other hand, in all groups thymectomized before 40 days, lymphatic leukemia occurred in only a very few cases. These results indicate that, although thymus had resided in the body up to 40 days, its removal was still capable of preventing the development of lymphatic leukemia in most animals. Therefore, it could be assumed that more than 40 days of continuous thymic function are necessary to allow transformation of lymphoid cells by the virus.

Reimplantation of thymus from 7 to 40 days after thymectomy was effective in restoring the tendency of animals to develop lymphatic leukemia. Nevertheless, in 35 to 43% of mice (all of which bore a recoverable thymus graft) myeloid leukemia occurred in spite of thymus implantation. This indicates that the absence of thymus for as few as 7 days was sufficient to permit malignant changes of myeloid cells. On the other hand, inadequacy or delay in the reconstitution of anatomical and functional properties of the implanted thymus could account for the partial failure to re-establish the tendency to lymphatic leukemia.

In our experiments, as in those by others (*13*, *20*), the susceptibility to lymphatic leukemia induction was not restored by thymus grafts in diffusion chambers. This does not necessarily mean that a thymic humoral factor is not involved in leukemogenesis. The short survival of thymic tissue in the chambers (*21*), while adequate to restore depressed immunological responsiveness (*21*, *22*), simply could not be sufficient to produce all the humoral substance needed for lymphatic leukemogenesis.

In about half the cases of myeloid leukemia developing in thymectomized, thymus-implanted mice of the various groups, the grafts showed neoplastic changes of lymphoid type. This finding confirms our previous observations on autonomous neoplastic transformation of thymus *in situ* in C57BL mice with generalized virus-induced myeloid leukemia. A possible explanation for the peculiar behavior of thymic tissue in these mice is that thymus possesses a special environment that prevents the development of malignant clones other than lymphatic within the organ itself. Whether the confinement of neoplastic lymphoid cells within the thymus in generalized myeloid leukemia depends on differences in the time required for malignant transformation or in proliferative kinetics of the two cell populations remains to be determined.

SUMMARY

Groups of (C3Hf/Gs × CBA/H)F_1 hybrid mice, inoculated at birth with cell-free extract containing Graffi virus, were thymectomized at various intervals after virus inoculation. In accordance with previous results obtained in other mouse strains, the removal of the thymus favored to a considerable extent a shift of neoplastic transformation from lymphoid to myeloid tissue. Thymectomy performed at 60 days of age was less effective in preventing lymphatic leukemia than if performed at 40 days or earlier.

Implantation of newborn C3Hf/Gs thymus, at various intervals from thymectomy in other groups of virus-inoculated hybrids, restored, at least partially, the tendency of thymectomized animals to lymphatic leukemia development. No restoring effect was found by grafting thymus in diffusion chambers.

In about half the thymectomized, thymus-grafted mice developing myeloid leukemia in spite of thymus implantation, the grafts showed neoplastic changes of lymphoid type. This finding confirms the autonomous neoplastic behavior of thymic tissue already observed in mice with generalized virus-induced myeloid leukemia.

The possible implications of these results on the mechanisms of thymus-dependency in Graffi virus leukemogenesis are discussed.

REFERENCES

(*1*) Fiore-Donati, L., and Chieco-Bianchi, L.: Influence of host factors on development and type of leukemia induced in mice by Graffi virus. J Nat Cancer Inst 32: 1083–1107, 1964.

(*2*) Chieco-Bianchi, L., Fiore-Donati, L., Tridente, G., and Pennelli, N.: Lymphatic neoplastic transformation of thymus in mice with myeloid virus-induced leukaemia. Nature (London) 208: 697–698, 1965.

(*3*) Osoba, D., and Miller, J.F.A.P.: The lymphoid tissues and immune responses of neonatally thymectomized mice bearing thymus tissue in millipore diffusion chambers. J Exp Med 119: 177–194, 1964.

(*4*) Levy, J. P., Lasneret, J., Peries, J., and Boiron, M.: Paralysies révélatrices de la leucémie de Gross chez le rat. Experientia 20: 627–628, 1964.

(*5*) Fiore-Donati, L., Chieco-Bianchi, L., Tridente, G., and Mazzarella, L.: Induction of malignant lymphomas by urethane in adult mice bearing syngeneic thymus grafts. Nature (London) 208: 398, 1965.

(*6*) Furth, J.: Prolongation of life with prevention of leukemia by thymectomy in mice. J Geront 1: 46–52, 1946.

(*7*) Gross, L.: Development of myeloid (chloro-) leukemia in thymectomized C3H mice following inoculation of lymphatic leukemia virus. Proc Soc Exp Biol Med 103: 509–514, 1960.

(*8*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*9*) Gross, L.: Pathogenic potency and host range of the mouse leukemia virus. Acta Haemat (Basel) 29: 1–15, 1963.

(*10*) ———: Serial cell-free passage in rats of the mouse leukemia virus. Effect of thymectomy. Proc Soc Exp Biol Med 112: 939–945, 1963.

(*11*) Kunii, A., and Furth, J.: Inhibition of lymphoma induction in virus-infected rats by thymectomy. An affinity of the lymphoma virus for myeloid cells. Cancer Res 24: 493–497, 1964.

(*12*) Nakakuki, K., and Nishizuka, Y.: Effect of adult thymectomy on lymphoid tissues and the pathology of leukemia in mice of high-leukemia strains. Gann 55: 509–520, 1964.

(*13*) Metcalf, D., Wiadrowski, M., and Bradley, R.: Analysis of the role of the thymus in leukemia development with the use of thymus grafts and thymectomy. Nat Cancer Inst Monogr 22: 571–586, 1966.

(*14*) Siegler, R., and Rich, M. A.: Pathogenesis of murine leukemia. Nat Cancer Inst Monogr 22: 525–547, 1966.

(*15*) Upton, A. C., Wolff, F. F., Furth, J., and Kimball, A. W.: A comparison of the induction of myeloid and lymphoid leukemias in X-radiated RF mice. Cancer Res 18: 842–848, 1958.

(*16*) Furth, J.: Influence of host factors on the growth of neoplastic cells. Cancer Res 23: 21–34, 1963.

(*17*) Miller, J.F.A.P., Block, M., Rowlands, D. T., Jr., and Kind, P.: Effect of thymectomy on hematopoietic organs of the opossum "embryo." Proc Soc Exp Biol Med 118: 916–921, 1965.

(*18*) Parrott, D. M. V., and East, J.: Role of the thymus in neonatal life. Nature (London) 195: 347–348, 1962.

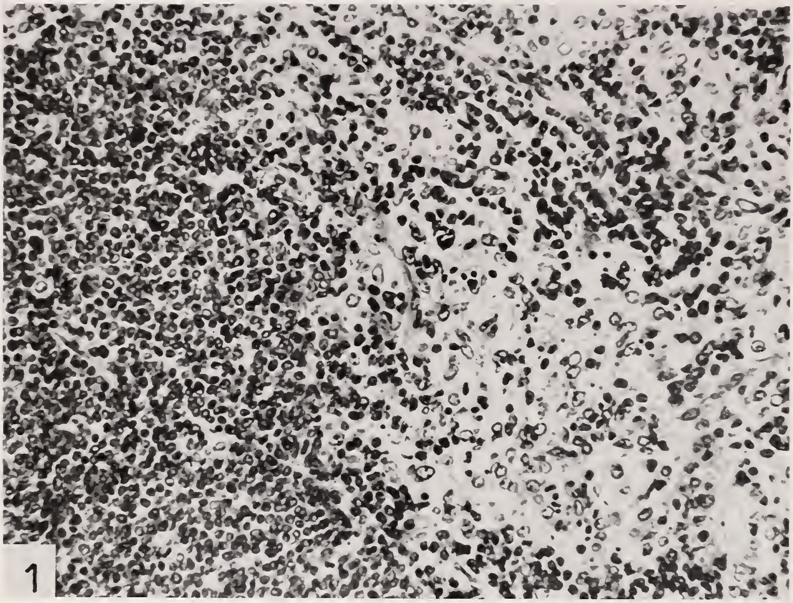
(*19*) Law, L. W., Dunn, T. B., Trainin, N., and Levey, R. H.: Studies of thymic function. *In* The Thymus (Defendi, V., and Metcalf, D., eds.). Philadelphia, The Wistar Institute Press, 1964, pp 105–117.

(*20*) Miller, J.F.A.P.: Role of the thymus in virus-induced leukaemia. *In* Ciba Foundation Symposium on Tumour Viruses of Murine Origin (Wolsten-Holme, G. E. W., and O'Connor, M., eds.). London, Churchill, 1962, pp 262–279.

(*21*) Levey, R. H., Trainin, N., and Law, L. W.: Evidence for function of thymic tissue in diffusion chambers implanted in neonatally thymectomized mice. Preliminary report. J Nat Cancer Inst 31: 199–217, 1963.

(*22*) Osoba, D., and Miller, J.F.A.P.: Evidence for a humoral thymus factor responsible for the maturation of immunological faculty. Nature (London) 199: 653–654, 1963.

PLATES

1


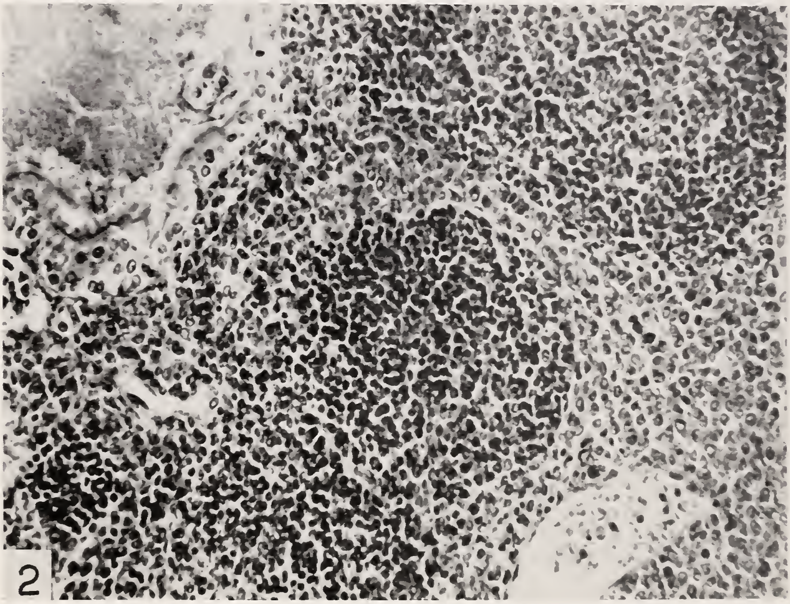
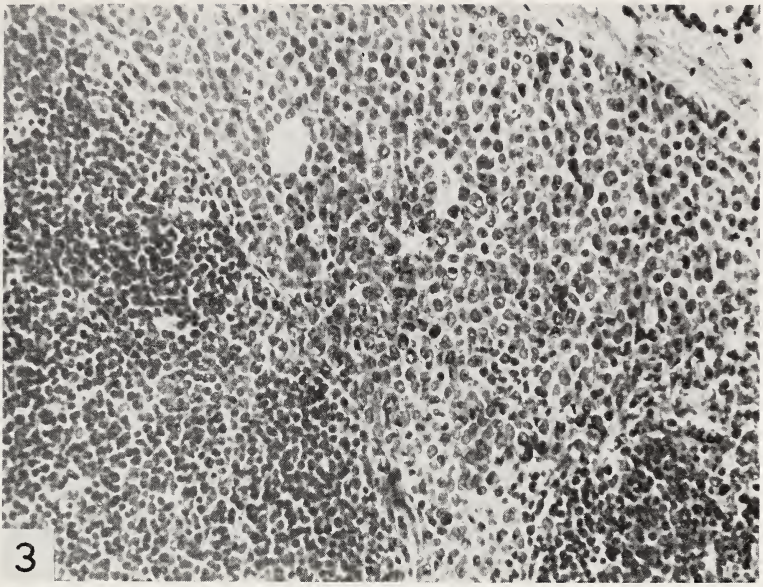
2


FIGURE 1.—Thymus graft of a mouse with lymphatic leukemia, implanted with a single thymus 7 days after thymectomy. The graft is composed of neoplastic lymphoid cells with islands of medullary tissue. Hematoxylin and eosin. × 320

FIGURE 2.—Thymus graft of a mouse with myeloid leukemia, implanted with a single thymus 20 days after thymectomy. Immature myeloid cells infiltrate partially the neoplastic lymphoid tissue of the graft. A cyst lined with ciliated epithelium is included in the field. Hematoxylin and eosin. × 320



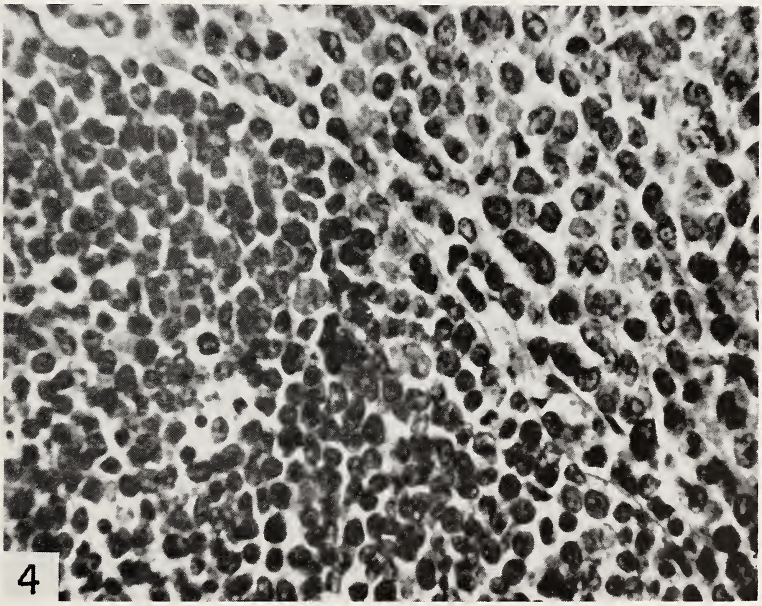


FIGURE 3.—Another example of myeloid leukemia with lymphatic neoplastic transformation of the thymus graft, in a mouse implanted with a single thymus 40 days after thymectomy. Hematoxylin and eosin. × 320

FIGURE 4.—Higher magnification of figure 3. Lymphoid cells on the *left*, myeloid cells on the *right*. Hematoxylin and eosin. × 720

DISCUSSION

Dr. Gross: Experiments carried out in our laboratory gave results similar to those reported by Dr. Fiore-Donati. We obtained the Graffi virus strain through the courtesy of Dr. Klein and studied it in our laboratory by inoculating suckling, less than 6 days old, mice of strains C3Hf and BALB/c. The form of leukemia induced with Graffi virus strain in C3Hf mice was predominantly lymphatic (75%) and stem cell (25%) ; there was no myeloid leukemia in this series. Among the BALB/c mice inoculated with the Graffi virus, 58% developed lymphatic leukemia, 3% had stem cell leukemia, and 27% developed the myeloid form. Inoculation of our Passage A virus into BALB/c mice also resulted in a similar or even higher incidence of myeloid leukemia. The Graffi virus did not, therefore, differ in its pathogenic potential from the Passage A virus. As a point of interest, I should like to mention that some of our mice with induced myeloid leukemia also had thymic lymphoid tumors; thus, we had both myeloid and lymphoid forms in the same host in a few cases.

Dr. Graffi (translated by Dr. Kirsten) : With the Graffi type of murine leukemia virus we have seen granulocytic, that is, myeloid leukemias in AKR mice.

Chairman Furth: In the same animals?

Dr. Graffi (translated by Dr. Kirsten) : No, we have not seen both forms of leukemia in the same animal. We believe this might have something to do with the function of the thymus, as we have just heard. We believe that the four strains of mice we are working with have what can be called a "weak thymus" so constitutionally they are more inclined to develop myeloid leukemia, or the conditions under which the animals are kept may favor the development of a myeloid leukemia. Perhaps there is another infectious agent which we will re-examine in the future.

Dr. Gross: My understanding of Dr. Graffi's remarks in German was that there may be some latent infection in his mice affecting the thymus; the effect of such infection may be comparable to "thymectomy" and may influence the form of induced leukemia.

Dr. Metcalf: I was fascinated by your results in which multiple thymus grafts increased the incidence of lymphoid leukemia. We have attempted to do a similar sort of thing using multiple C57BL grafts in C57BL mice but failed to increase the incidence of leukemia. I think it is becoming apparent from this meeting and others that most viruses may not induce neoplasia directly as a primary effect but that neoplastic change occurs in a *viral-damaged* cell population. If this is true, then the more cells you have at risk the more likely such a subsequent event will occur. Your experiment shows this very nicely.

Chairman Furth: In Kunii's experiment, as in yours, the incidence of leukemia was directly related to the number of thymuses grafted. Correspondingly, the lymphocyte levels were increased in direct relation to the number of thymuses (up to 10) grafted. (Cancer Res 1966, Jan., and Ciba Symposium on the Thymus. In press)

Dr. Kirsten: I have a question for Dr. Lieberman, Dr. Metcalf, and Dr. Fiore-cells were gradually replaced by host cells. Dr. Metcalf, working with a T6 hybrid, transfer. By using sex chromosome as markers the homologously transplanted cells were gradually replaced by host cells. Dr. Metcalf, working with a T6 hybrid, have you found whether these cells are of host or donor origin by chromosomal analyses?

Dr. Fiore-Donati: We made our experiments in a hybrid combination (C3Hf/Gs × CBA/H)F_1, in which we could investigate by chromosome analysis the origin of cells in the neoplastic graft. On the basis of preliminary results, in most mice neoplastic cells in the graft were of host origin.

I would add a comment on the paper of Professor Graffi. In our laboratory we have never observed myeloid leukemia in AKR mice inoculated at birth with Graffi virus. AKR mice injected with this virus developed almost exclusively lymphatic leukemia, which appeared much earlier than in noninoculated, control mice. This was regarded as a phenomenon of superinfection comparable to the acceleration observed by Rudali *et al.* (Bull. Assoc. Franc. Cancer 44: 440–443, 1957) in AKR mice inoculated at birth with Gross virus. We obtained a high incidence of myeloid leukemia in AKR mice, as well as in C3H mice, only when the thymus was previously removed (Fiore-Donati, L., and Chieco-Bianchi, L., J. Nat. Cancer Inst. 32: 1083–1107, 1964).

Dr. Reif: With regard to a marker, we have reported on two thymic antigens differing from the thymus-leukemia antigen TL reported by Old, Boyse, and Stockert. Either one or the other of the former two thymic antigens is present in all strains of mice we have so far examined. AKR and RF mice possess one of these antigens; all other strains examined possess the other. Since these antigens are strong, they are easy to detect. I believe they could be used as markers in several of the studies that have been described. For instance in Dr. Metcalf's elegant experiments, he could have differentiated in conjunction with the fluorescent antibody technique. Dr. Donati also could have effectively used these cellular antigens as markers in his experiments.

Dr. Fiore-Donati: In conclusion, our experiments also show that in leukemogenesis by Graffi virus the thymus plays an essential role in the development of lymphatic leukemia. It is still uncertain whether the removal of thymus favors the development of myeloid leukemia also, indirectly by abolishing a possible control action of this organ on myeloid tissue.

The Thymus Autoimmunity and Malignancy in New Zealand Black Mice [1]

June East *and* Maria A. B. de Sousa, *Imperial Cancer Research Fund, Mill Hill, London, N.W. 7, England*

New Zealand Black (NZB) mice, unlike those of other strains, develop an autoimmune hemolytic anemia (*1*) and proliferative lesions (germinal centers) in the thymic medulla (*2*). The autoimmune reaction is manifest as a positive antiglobulin (Coombs) test (*3*), and it has been suggested that the thymic germinal centers are the production sites of autoantibody-producing cells (*4*). The autoantibody coating the red cells has been identified as 7Sγ-globulin by Norins and Holmes (*5*) or as 19S-macroglobulin by Mellors (*6*), while Holborow *et al.* (*7*) have described another autoantibody reactive with antigenic sites accessible in the stromata of both human and mouse cells.

In a previous study of the immunopathology of the NZB mice (*8*), we drew attention to the anomalies existing between the presence of the thymic lesions, the occurrence of a positive Coombs test, and the clinical condition of the animals. We also reported that the animals were macroglobulinemic. It is our intention to summarize these data, to present new information obtained from transfer experiments, and to discuss some hypotheses concerning the thymic lesions, the immunologic status of the NZB mice, and the malignant changes occurring in their lymphoid organs.

RESULTS

Postmortem Findings

Most NZB mice appeared normal until they were about 24 weeks old when the males, in particular, began to show alopecia, ulceration, and scabbing of shoulders, back, and hindquarters; these conditions were exacerbated by the tendency of the males to fight viciously. However, some (7%) of the younger animals developed transplantable lymphocytic thy-

---

[1] **Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.**

Table 1.—Postmortem findings in NZB mice

Age (weeks)	Number of mice	Number with thymomas	Number with spleen and/or lymph nodes:	
			Normal	Enlarged
12–23	4 ♀	1	2	1
	4 ♂	2	1	1
24–31	3 ♀	1	2	—
	5 ♂	1	4	—
32–43	14 ♀	3	7	4
	9 ♂	—	4	5
44–77	49 ♀	—	3	46
	31 ♂	—	4	27
	70 ♀	5 ♀	14 ♀	51 ♀
	49 ♂	3 ♂	13 ♂	33 ♂

momas that produced lethal, generalized lymphocytic leukemia 14 to 18 days after intraperitoneal injection into 1- or 28-day-old syngeneic recipients. Most mice died after the age of 32 weeks (table 1). Analysis of data on 119 mice, in addition to that already published (*8*), shows that males and females die, on the average, at the same time, namely, 48 weeks (males 48.6 weeks, range 15–75 weeks; females 48.4 weeks, range 17–77 weeks). The most striking postmortem findings in both sexes were splenomegaly and lymphadenopathy (fig. 1). The spleen was often grossly enlarged but, although the superficial nodes attained considerable size, the mesenteric node was not so consistently affected. The thymus, was, however, small or atrophic. Hepatosplenomegaly occurred in approximately 15 percent of the mice, most often in the females. Discoloration or hemorrhagic patches were seen in the lungs of older mice of both sexes. An additional fact, not previously reported, was the occurrence of prostatitis in older males, some of which also had infections of the bladder and kidneys.

Hematological Findings

White cell counts.—Mice in the terminal stage of illness had massive polymorphonuclearcytosis (table 2). The lymphocyte: polymorph ratio of younger animals also differed from that of other strains of mice with which we are familiar. In the NZB strain the lymphocytes and polymorphs occurred in approximately equal proportions and not in the more usual 70: 30 ratio. A few of the older, sick mice were severely lymphopenic. The monocyte population also tended to be high at all ages and some mice, although apparently healthy, had a definite monocytosis. Other types of cells, not often seen in normal blood, were identified in the smears of NZB mice, and their numbers increased as the animals aged. They could be reticulum cells, plasma cells, or primitive undifferentiated cells that we were unable to classify.

TABLE 2.—Leukocyte counts of NZB mice

Number of mice	Age (days)	Number of cells/mm^3—mean and range					Percentage cells—mean and range			
		Total	Lymphocytes	Polymorphs	Monocytes	Others*	Lymphocytes	Polymorphs	Monocytes	Others*
10 5♀5♂	33–138	6109 (1800–9100)	3173 (1729–5506)	2384 (504–4346)	537 (54–1020)	15 (9–46)	52. 0 (41. 0–68. 5)	38. 6 (23. 0–53. 0)	9. 0 (2. 0–12. 5)	0. 4 (0. 5–1. 0)
10 4♀6♂	a)98–182	5302 (1266–11, 800)	1679 (717–2435)	3110 (456–7788)	378 (53–826)	135 (22–708)	37. 3 (20. 0–68. 0)	53. 4 (12. 0–78. 0)	7. 3 (2. 0–15. 0)	2. 0 (0. 5–6. 0)
6 4♀2♂	b)191–302	4759 (3550–7000)	2211 (1630–3535)	2147 (899–2835)	264 (87–455)	137 (40–400)	46. 2 (39. 0–57. 3)	44. 7 (25. 3–59. 0)	5. 8 (2. 0–12. 7)	3. 3 (1. 0–11. 0)
11 6♀5♂	335–511†	13, 795 (2350–21, 400)	3101 (235–6630)	8703 (1914–17, 762)	1103 (67–2414)	888 (63–3010)	22. 2 (10. 5–51. 0)	62. 7 (23. 5–88. 8)	9. 2 (1. 0–25. 5)	5. 9 (0. 5–14. 0)

*Includes reticulum cells, plasma cells, and primitive undifferentiated cells.
†Mice bled on day of death.

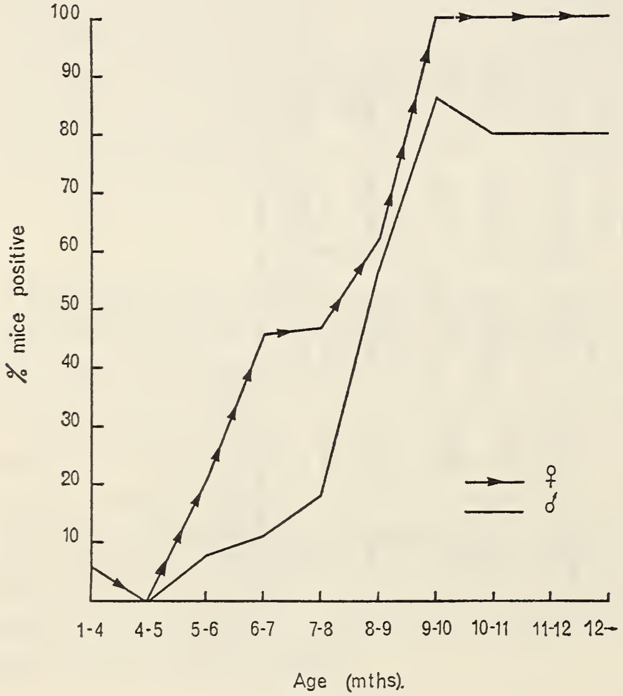
TABLE 3.—Hematocrits of NZB mice

Number of mice	Age (days)	Hematocrit value (%—mean and range)
3 ♀	45–97	45. 3 (42. 5–48. 8)
3 ♂	45–97	45. 7 (44. 0–48. 0)
6 ♀	371–535*	23. 3 (14. 0–38. 0)
5 ♂	285–532*	27. 0 (22. 0–38. 0)

* Mice bled on day of death.

Hematocrits.—Although low hematocrit values (mean 25%) were recorded for many mice bled on the day of death, not all were severely anemic (table 3).

Direct antiglobulin (Coombs) tests.—The results of 295 direct antiglobulin tests performed on tail blood samples taken from 139 mice are shown in text-figure 1. Animals were only classed as positive if their tests scored + or more in at least 2 of the 3 dilutions of antiserum (Microbiological Associates, Bethesda, Maryland) used, namely, 1/5, 1/25, and 1/125. The first positive, but weak, reactions were found in both males and females at 20 to 23 weeks and thereafter increased in intensity. In our colony, the female mice developed positive Coombs tests in advance of the males, and a small number of the latter were still negative at death. There is, therefore, no close correlation between the clinical condition of the mice and the development of positive reactions; some healthy animals were positive at 5 months while other, much older mice remained negative until they were already deteriorating.



TEXT-FIGURE 1. — Incidence of positive direct antiglobulin tests in NZB mice.

Histological Findings

In a preliminary survey of 80 animals age 1 day to 59 weeks, we found proliferative thymic lesions, apparently similar to the germinal centers of the spleen and lymph nodes, in only a relatively few (14/80) individuals (*8*). Further, these lesions were detected in young mice killed as healthy controls as often as in old, sick mice. The lack of correlation between the presence of thymic germinal centers, the development of positive Coombs tests, and the clinical condition of the mice led us to investigate more closely the histology of all the lymphoid organs—spleen and lymph nodes as well as thymus.

Two distinct histological phases were distinguished in the spleen and lymph nodes. The first, exhibited by mice 3 to 11 months old, was a phase of lymphoid hyperplasia in the white pulp of the spleen and in the cortex of the lymph nodes. It was characterized by large, hyperactive follicles with multiple germinal centers. The second phase occurred from 11 months onward and was typified by extensive proliferations of pyroninophilic cells (large pyroninophilic lymphoid cells, immature and mature plasma cells) and/or reticulum cells in the red pulp of the spleen and in the medullary cords of the lymph nodes. The thymus at this time was either well organized but contained sheets of pyroninophilic cells or was atrophic with a narrow cortical rim and a hypocellular hyaline medulla.

In the latter part of the first or lymphoid phase, periodic acid-Schiff-positive globules were distinguished in sections of the spleen and lymph nodes and cells, which had characteristics of both lymphocytes and plasma cells (the "lymphoid-plasma cells"), were found in impression smears of the organs. The association of these occurrences in macroglobulinemic patients prompted immunoelectrophoretic analyses of the sera of NZB mice. These analyses were performed by Dr. John Humphrey of the National Institute for Medical Research, London, using rabbit-anti-normal mouse IgM (γ_1M-, β_2M-globulin) antiserum provided by Dr. John Fahey of the National Institutes of Health, Bethesda, Maryland. The analyses showed that the mice produced large amounts of 19S macroglobulin (IgM) irrespective of their age, sex, clinical condition, antiglobulin reaction, or histological state (*8*).

Effects of Neonatal Thymectomy

Removing the thymus from NZB mice within 12 to 72 hours after birth does not prevent, but may even precipitate, the appearance of a positive Coombs test. Within 6 to 8 weeks, neonatally thymectomized mice developed the same lethal wasting syndrome described for mice of other strains (*9*, *10*). Their blood lymphocytes were severely reduced and specific areas of lymphocyte depletion—the thymus-dependent areas (*11*)—were seen in the mid cortex and deep cortex of their lymph nodes.

However, the diminution of the splenic follicles and germinal centers, which follows neonatal thymectomy in other strains, did not occur in NZB mice. On the contrary, the germinal centers remained prominent and active and contained numerous large pyroninophilic lymphoid cells, cells in mitosis, and cellular debris.

Transfer Experiments

The prolific distribution of the reticulum and plasma cells in the old NZB mice suggested malignant change, and for this reason, a series of passage experiments was initiated. These experiments also enabled us to test the possibility that cells capable of producing autoantibody could be transferred to syngeneic recipients and to determine whether a transmissible (? viral) agent was involved.

Cell suspensions prepared from the enlarged spleens or lymph nodes of 5 sick, female, Coombs-positive donors, age 47 to 76 weeks, were passaged serially by intraperitoneal injection in either intact or neonatally thymectomized NZB mice age 1 to 76 days (table 4). The first recipients of the donor material developed, within 7 to 30 weeks of injection, very much larger spleens than controls of the same age. Some of these recipients remained healthy for 41 weeks despite having spleens that could be easily palpated, but others were moribund when killed. However, by the third passage all the recipient mice became extremely ill only 3 to 7 weeks after injection and had massive spleens weighing 1 to 2 g when autopsied. The condition of the lymph nodes varied from series to series but it was consistent throughout each passage. In one series all the nodes were enlarged and hemorrhagic; in others, the mesenteric node was unaffected. The thymus was never involved and remained macroscopically normal in size and color. Detailed histological examination of tissues taken from the recipients is still in progress, but a preliminary study of impression smears of bone marrow, spleen, and nodes indicates that the proliferating elements were large or medium-sized, very primitive, reticulum cells. Similar cells were also present among the more heterogeneous populations of the original donors' cells.

In three other experiments, transfer of bone marrow, ascitic fluid, or spleen tissue from, respectively, 2 female and 1 male Coombs-positive donors, age 37 to 65 weeks, was without effect. We previously reported (*8*) also that spleen or thymus cell suspensions from young Coombs-negative donors did not affect syngeneic or allogeneic recipients. In no instance did the lymphoid tissues from the Coombs-positive donors, whether passaged successfully or unsuccessfully, provoke positive antiglobulin reactions in the young NZB recipients. We find this difficult to understand, since other workers (*7*, *12*) have transferred the antiglobulin reaction by a similar method, although they made no reference to malignant proliferation.

TABLE 4.—Passage of cell suspensions of the lymphoid organs of old NZB donors in young NZB recipients

Age of donor	Passage number	Cell suspension	Type and age of recipients	Number of mice dying / Number of mice injected	Period post injection (days)
A 50 weeks	1	a) Spleen	Intact—1 day	3/4	103–199
	2	a) Spleen	Intact—21 days	0/2	—
			Intact—76 days	1/2	96
	3	a) Spleen	Intact—1 day	5/5	30–32
B 57 weeks	1	a) Spleen + lymph node	Intact—1 day	2/5	122, 156
	2	a) Spleen	Intact—4 days	3/3	51–65
		b) Lymph node	Intact—4 days	2/2	62, 72
	3	a) Spleen	Intact—2 days	2/2	45, 48
		b) Lymph node	Intact—6 days	6/6	41–48
C 76 weeks	1	a) Spleen	Intact—18 days	2/2	49*, 69
		b) Lymph node	Thymectomized—18 days	2/2	63*, 85
	2	a) Spleen	Thymectomized—7 days	4/4	25–38
		b) Spleen	Intact—4 days	4/5	21–34
	3	a) Spleen	Thymectomized—10 days	4/4	18–25
D 56 weeks	1	a) Spleen	Intact—1 day	2/4	185*, 214
	2	a) Spleen	Thymectomized—2 days	2/2	42, 60
E 47 weeks	1	a) Spleen	Intact—26 days	3/7	139–177
	2	a) Spleen	Thymectomized—15 days	3/3	32–43

*Killed healthy with enlarged spleens as donors for subsequent passage.

Cell-free filtrates of passage material have been injected into newborn intact or thymectomized syngeneic or allogeneic recipients, but the results are not yet available.

DISCUSSION

Although NZB mice have proliferative lesions in the thymic medulla, develop a Coombs-positive hemolytic anemia, and die before they are 77 weeks old, having gross enlargement of the spleen and lymph nodes, there is no close correlation between these events. We have now demonstrated that these mice are macroglobulinemic and that the splenomegaly and lymphadenopathy are due to a massive proliferation of plasma and/or reticulum cells. Our transfer experiments could indicate that primitive reticulum-type cells can be selected out from the spleens and lymph nodes of old, sick donors by serial passage in young NZB mice and that these cells proliferate quickly and extensively in the lymphoid tissues of the recipients. That the recipients do not develop positive antiglobulin reactions would, in this case, suggest that the proliferating cells are not producing autoantibody. It also will be extremely interesting to determine whether they are synthesizing 19S macroglobulin. On the other hand, the experiments could indicate that a transmissible agent carried by the donor cells had provoked proliferation of the recipient's own cells.

A number of working hypotheses can be erected on the basis of the data presented. The fact that NZB mice develop positive Coombs tests in the absence of the thymus suggests that the proliferative lesions occurring in the thymus of the intact animals are not the cause of the autoimmune condition but the consequence of occurrences elsewhere in the body. The spectrum of histological changes seen in the spleen and lymph nodes are, albeit in a lesser degree, characteristic responses to antigenic stimulation, and it may be that NZB mice are under extreme endogenous antigenic pressure. This pressure could be of a viral nature but it then becomes necessary to explain why reactions are elicited in the thymus, which is normally sacrosanct in this respect. Here it may be relevant to remember that Aleutian disease of mink, which is characterized by systemic proliferation of plasma cells and by hypergammaglobulinemia, is now believed to have a viral etiology (*13–16*). Alternatively, the NZB mice could be immunologically hyper-reactive and be responding in an exaggerated fashion to normal, exogenous, antigenic stimuli. Finally, the extraordinary proliferation of both plasma and reticulum cells in NZB mice may indicate a defect in the mechanism by which the balance of cell populations is controlled, and there is evidence that this control is normally exercised by the thymus. For instance, it is known that plasma and reticulum cells proliferate in the lymphocyte-depleted lymphoid organs of neonatally thymectomized mice of other strains (*9*, *17*), that such animals produce increased amounts of another immunoglobulin γ-1A

(*18*), and that their immunological responses to certain humoral antigens are unimpaired (*18*). If this last hypothesis is correct, then the autoimmune hemolytic anemia, the macroglobulinemia, and the malignant proliferation of plasma or reticulum cells in NZB mice may all be consequences of a basic thymic insufficiency.

REFERENCES

(*1*) Bielschowsky, M., Helyer, B. J., and Howie, J. B.: Spontaneous haemolytic anaemia in mice of the NZB/BI strain. Proc Univ Otago Med Sch 37: 9, 1959.

(*2*) Burnet, F. M., and Holmes, M. C.: Immunological function of thymus and bursa of Fabricius. Thymus lesions in an autoimmune disease of mice. Nature (London) 194: 146–147, 1962.

(*3*) Holmes, M. C., and Burnet, F. M.: The natural history of autoimmune disease in NZB mice. Ann Intern Med 59: 265–276, 1963.

(*4*) Burnet, F. M.: Role of the thymus and related organs in immunity. Brit Med J 2: 807–811, 1962.

(*5*) Norins, L. C., and Holmes, M. C.: Globulins on NZB mouse erythrocytes. J Immun 93: 897–901, 1964.

(*6*) Mellors, R. C.: Autoimmune disease in NZB/BL mice. I. Pathology and pathogenesis of a model system of spontaneous glomerulonephritis. J Exp Med 122: 25–40, 1965.

(*7*) Holborow, E. J., Barnes, R. D. S., and Tuffrey, M.: A new red-cell autoantibody in NZB mice. Nature (London) 207: 601–604, 1965.

(*8*) East, J., de Sousa, M. A. B., and Parrott, D. M. V.: Immunopathology of New Zealand Black (NZB) mice. Transplantation 3: 711–729, 1965.

(*9*) Parrott, D. M. V., and East, J.: Role of the thymus in neonatal life. Nature (London) 195: 347–348, 1962.

(*10*) ———: Studies on a fatal wasting syndrome of mice thymectomized at birth. *In* The Thymus in Immunobiology (Good, R. A., and Gabrielson, A. E., eds.). New York, Hoeber-Harper, chapt 29, 1964.

(*11*) Parrott, D. M. V., de Sousa, M. A. B., and East, J.: Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. J Exp Med 123: 191–204, 1966.

(*12*) Holmes, M. C., Gorrie, J., and Burnet, F. M.: Transmission by splenic cells of an autoimmune disease occurring spontaneously in mice. Lancet 2: 638–639, 1961.

(*13*) Helmboldt, C. F., and Jungherr, E. L.: The pathology of Aleutian disease in mink. Amer J Vet Res 19: 212–222, 1958.

(*14*) Henson, J. B., Gorham, J. R., Leader, R. W., and Wagner, B. M.: Experimental hypergammaglobulinaemia in mink. J Exp Med 116: 357–364, 1962.

(*15*) Kenyon, A. J., Trautwein, G., and Helmboldt, C. F.: Characterization of blood serum proteins from mink with Aleutian disease. Amer J Vet Res 24: 168–173, 1963.

(*16*) Henson, J. B., Gorham, J. R., and Leader, R. W.: Hypergammaglobulinaemia in mink initiated by a cell-free filtrate. Nature (London) 197: 206–207, 1963.

(*17*) East, J., and Parrott, D. M. V.: Prevention of wasting in mice thymectomized at birth and their subsequent rejection of allogeneic leukemic cells. J Nat Cancer Inst 33: 673–690, 1964.

(*18*) Humphrey, J. H., Parrott, D. M. V., and East, J.: Studies on globulin and antibody production in mice thymectomized at birth. Immunology 7: 419–439, 1964.

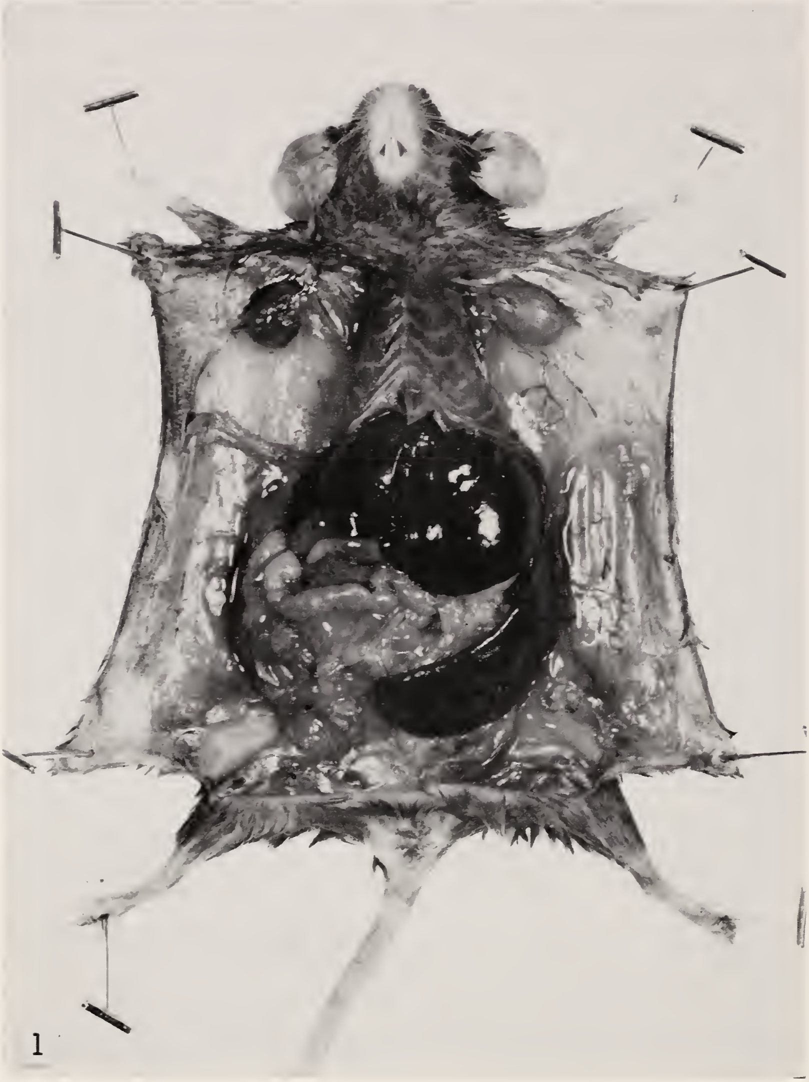


FIGURE 1.—Postmortem appearance of an NZB female mouse killed when sick at 34 weeks of age. *Note* enlarged spleen and lymph nodes.

DISCUSSION

Dr. Metcalf: I agree with Dr. East. I don't think anyone now believes that the thymic proliferative lesions are related etiologically to the autoimmune hemolytic anemia, although there is some evidence from Helyer and Howie in New Zealand that thymus grafting from the New Zealand mouse into the thymectomized F_1 hybrid may induce an immune disease (Howie, J. B., and Helyer, B. J., Ann. N.Y. Acad. Sci. 124: 167, 1965).

Just one question. You may have said this but I missed it: What cells were you transplanting in those passage lines?

Dr. East: In fact, spleen or lymph node cells—cell suspensions, very concentrated.

Dr. Metcalf: These were *cell* transfer studies going on in Dr. Holmes' laboratory and if I remember correctly she also observed splenomegaly in the first-passage mice, which was minor in degree, but I don't believe she has tried the effect of serial passage on this splenomegaly effect.

Dr. East: I think her report concerned 4 animals and although the splenomegaly was quite considerable, it was not as massive as ours. The interesting fact is that Dr. Holmes' recipients became Coombs positive. We just can't make ours become Coombs positive after transfer of cells. It seems to me simply to indicate that we are not transferring the autoantibody-producing cells. But I don't understand the difference.

Chairman Furth: Did you ever get a virus out of this?

Dr. East: We are presently experimenting using cell-free extracts and filtrates within and across strain.

Dr. Dunn: I just want to express appreciation for this very clear description. When Dr. Bielchowsky was in the United States about 3 years ago, he showed me many sections from some of his NZB mice, and we concluded that many of the lesions represented what we very loosely call the Hodgkins' disease-like lesion of mice. It would be of considerable interest if it is this type of neoplasm that is being successfully transplanted. In our experience, this tumor does not often undergo successful transplantation.

Dr. Rappaport: This is a most exciting and interesting paper, particularly because of the histologic similarities between the murine and human diseases. In 1955, we reported periodic acid-Schiff-positive globules in lymphoma cells of patients who also had Coombs-positive autoimmune hemolytic anemia (Rappaport, H., and Johnson, F. B.: Intracellular protein resembling Russell bodies in malignant lymphomas associated with acquired hemolytic anemia. Blood 10: 132–144, 1955). Histochemically, these globules were glycoproteins. We also reviewed 65 malignant lymphomas and found in 7 of these periodic acid-Schiff-positive globules in the lymphoma cells. Since we were dealing with material submitted for consultation, Coombs tests were usually not available, but in some instances suggestive evidence of hemolytic anemia was present. Subsequently, Dutcher and Fahey described the pathology of macroglobulinemia and in reviewing our previously published material concluded that at least 2 of our cases probably were instances of macroglobulinemia (Dutcher, T. F., and Fahey, J. L.: The histopathology of the macroglobulinemia of Waldenström. J. Nat. Cancer Inst. 22: 887–918, 1959). It is well known that macroglobulinemia and autoimmune hemolytic anemia may coexist in man. The observation of similar morphologic manifestations of dysproteinemia in the mouse with autoimmune hemolytic anemia is of great interest.

Dr. Sinkovics: Dr. East, evidently, these cell suspensions do not cause runt disease.

Dr. East: No, because we are passaging in the syngeneic situation to young New Zealand Black mice. Is that what you meant?

Dr. Sinkovics: Yes, but you can break through that syngeneic situation if the cells are autoimmune.

Dr. Kirsten: I have a technical question. I suppose the idea of its being an autoimmune disease is based on the Coombs test. It is, however, known that animals with hemolytic anemia and many circulating reticulocytes develop a positive Coombs test which in this situation would not indicate autoimmunity. For example, in phenylhydrazine-induced anemia one can convert a negative to a positive Coombs test. Does the late development of the positive Coombs test in the table you showed not indicate that the animals have a hemolytic anemia that is not necessarily autoimmune?

Dr. East: As I said, we were interested primarily in the reactions of the lymphoid tissues and not so much in the anemia, and I must say I really don't know what to believe about this. We assumed this was an autoimmune situation—the classic situation as described by other workers. I am not certain in my mind that it is, particularly in view of this difference of opinion as to the nature of the coating antibody.

Dr. Block: Another technical point is that autoagglutination of red cells occurs in individuals with a macroglobulin. Autoagglutination of red cells may occur prior to addition of the Coombs reagent unless the red cells are carefully washed.

Dr. East: We very carefully washed the cells.

GENERAL SESSION

Chairman: FRANK J. RAUSCHER

Genetic Control of Susceptibility to Friend Leukemia Virus in Mice: Studies With the Spleen Focus Assay Method [1, 2]

A. Axelrad,[3, 4] *Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, Toronto, Canada*

It is well known that mice of various inbred strains have very different degrees of susceptibility to Friend virus (*1–9*). In 1962, Odaka and Yamamoto (*7*) presented evidence that the difference in susceptibility between two mouse strains, RF and C57BL/6, was determined mainly at a single autosomal gene locus, and that susceptibility was dominant over resistance. Their study was carried out by standard procedures in which susceptibility was scored on the basis of splenomegaly and elevated peripheral nucleated cell count, both signs of rather advanced disease.

We wished to re-examine the question of the genetic control of susceptibility to Friend virus with the help of the spleen focus assay method (*10*), which permits scoring for susceptibility relatively early in the course of the disease.

The spleen focus assay method is based on the fact that following the injection of appropriate doses of Friend virus into susceptible mice, focal lesions rapidly develop in the spleen. These lesions are visible to the naked eye on the surface of the intact spleen, and they can be counted. Because a linear relation exists between the dose of virus administered and the mean number of macroscopic foci present at 9 days after infection, the method provides a reliable and convenient means of titrating Friend virus infectivity in mice.[5]

In previous experiments (*12*, *13*) the method was used to detect variations in residual infectivity after known quantities of Friend virus had been subjected to various treatments; the method thus gave information

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This work was supported by grants from: the National Cancer Institute of Canada; U.S. Public Health Service research grant CA-4964-05, National Cancer Institute; and the Medical Research Council of Canada, grant MA-1565.

[3] With the technical assistance of H. C. van der Gaag.

[4] The author wishes to acknowledge the helpful advice of Dr. L. Siminovitch in the preparation of the manuscript and the able technical assistance of Mr. G. Mahaffy and Miss S. Dalley.

[5] The counterpart of this method for titrating infectivity of Rauscher virus in mice is based on a similar phenomenon and quantitative relation (*11*).

about properties of the virus. In the work to be described, the approach was reversed, and the method was used to detect variations in response to fixed doses of Friend virus by hosts of different genotype.

RELATIVE SUSCEPTIBILITY OF VARIOUS INBRED STRAINS OF MICE TO SPLEEN FOCUS INDUCTION BY FRIEND VIRUS

In order to find and quantify differences in Friend virus susceptibility among mice of different genotypes, which could then be used for further genetic analysis, a survey of 12 inbred strains of mice was carried out with the help of the spleen focus assay method.

Groups of young adult mice from each strain received graded doses of Friend virus intravenously, and the numbers of macroscopic foci in their spleens were scored 9 days later. For comparing host responses to the virus, a "susceptibility index" was determined for each strain (*14*). This index represented the initial slope of the line relating the number of macroscopic foci in the spleen to the dose of virus administered.[6]

The relative susceptibilities to Friend virus among the 12 mouse strains tested, in order of decreasing susceptibility from left to right, were: SWR/J > SIM/Mc > DBA/1J ≃ DBA/2J ≃ RF/J > > C3H/Bi ≃ C3H/DiSn ≃ AKR/J > > > A/WySn ≃ BALB/cJ > > C57BL/6JOci ≃ C57BL/10ScSn.

This order was in general, but not exact, agreement with that found by Fieldsteel *et al.* (*5*), Odaka and Yamamoto (*7*), and Old *et al.* (*9*) using standard methods for estimating susceptibility to Friend virus.

The susceptibility indexes of these mouse strains spanned a wide range. From among these strains, therefore, it was possible to select mice of very high and of very low susceptibility with which to carry out the appropriate crosses for further investigation of the mode of inheritance of Friend virus susceptibility. SIM/Mc (Swiss inbred mice) and C57BL/6JOci mice were chosen for this purpose, for their susceptibility indexes differed by a factor in the order of 10,000-fold.

INHERITANCE OF SUSCEPTIBILITY AND RESISTANCE TO SPLEEN FOCUS INDUCTION BY FRIEND VIRUS, AS DETERMINED BY CROSSES BETWEEN C57BL/6 AND SIM STRAINS

A comparison was made of the numbers of foci induced by graded doses of Friend virus in the spleens of C57BL/6JOci and SIM/Mc, and in the spleens of their reciprocal F_1 hybrids. The mean number of foci per spleen in the F_1 hybrid mice was intermediate between those of the parental strains at all virus doses tested. No significant differences were noted between reciprocal hybrids of either sex.

---

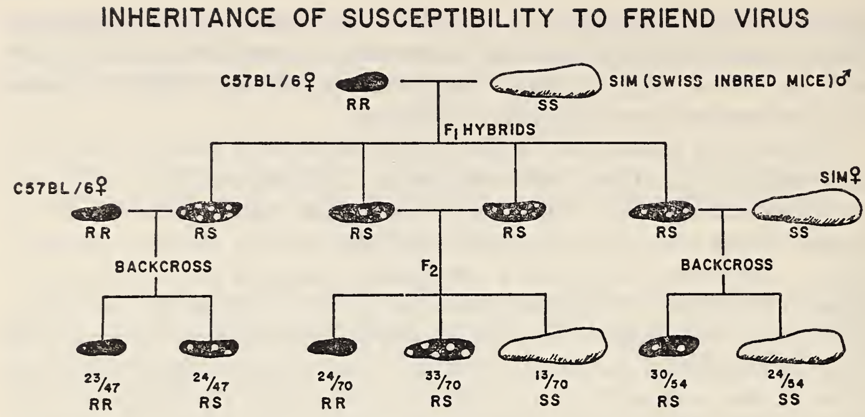
[6] Virus doses were expressed in "cell-equivalents," which represent dilutions of virus extracted from spleens of known cell number taken from Friend virus-infected mice with far-advanced disease. The virus used in all experiments described in this paper was cultivated in C3H/Bi mice.

C57BL/6, F_1 hybrid, and Swiss inbred mice could therefore be characterized as being of low, intermediate, and high susceptibility to Friend virus, respectively. To find out whether these traits segregated in further crosses, it was necessary to score progeny individually for their susceptibility. But there was usually a considerable range of focus numbers per spleen in animals of identical genotype given the same dose of virus; overlaps in focus number between groups could thus give rise to serious errors in classifying individual animals. From the experiment described above, it was evident that this difficulty could be met by choosing a high dose of virus. This had little effect on the spleens of C57BL/6 mice (fig. 1), and thus the spleens of F_1 hybrids, with discrete, readily countable foci (fig. 2) could be distinguished from them with little overlap. In animals of the SIM strain, the high virus dose produced greatly enlarged pale spleens with confluent foci too numerous to count (fig. 3), which were easily distinguishable from the spleens of F_1 hybrids. It thus appeared feasible to classify individual spleens, with little ambiguity, as being of C57BL parental type, of F_1 hybrid type, or of SIM parental type, in subsequent experiments. In taking this approach, the spleen focus assay method is used in a quite different way from that described in the other experiments. Although both functions of the method depend on its ability to discriminate between different degrees of susceptibility to Friend virus, the method serves here rather as a means of classifying than of quantifying responses to Friend virus.

A dose of Friend virus containing 5×10^6 cell-equivalents of virus was given to groups of mice of C57BL/6 and SIM parental strains, to (C57BL/6 × SIM)F_1 and F_2 hybrids, and to the progeny of backcrosses to both parental strains.

The results are shown in text-figure 1. Most of the F_2 hybrid spleens were in the 3 classes described above. The ratios among the 3 classes, 24:33:13, respectively, were not significantly different from the 1:2:1 ratios that would be expected on a single gene locus hypothesis where the two alleles both expressed themselves in the heterozygote ($\chi^2 = 3.38$; $0.1 > P > 0.05$). Data from backcrosses of F_1 hybrid males to C57BL/6 (BBC) and to SIM (SBC) parental strain females also agreed with expectation on the same hypothesis. The ratios of F_1 hybrid type to C57BL/6 parental type, 30:24, in the first, and of F_1 hybrid type to SIM parental type, 23:24, in the second, were not significantly different from 1:1 ($\chi^2_{\mathrm{BBC}} = 0.02$; $0.9 > P > 0.8$; $\chi^2_{\mathrm{SBC}} = 0.66$; $0.5 > P > 0.3$).

Phenotypically, then, the progeny of crosses, intercrosses, and backcrosses between C57BL and SIM mice behaved as if their susceptibility (or resistance) to spleen focus induction by Friend virus is determined



Text-figure 1.—Diagram showing effects of Friend virus on the spleens of young adult mice of strains C57BL/6 and SIM, their F_1 and F_2 hybrid progeny, and the progeny of backcrosses to both parental strains. Spleens, fixed in Bouin's fluid, were examined and classified 9 days after infection. The observed frequencies of spleens in the C57BL/6, F_1 hybrid or intermediate, and SIM classes are shown at *bottom* of the diagram. The ratios are consistent with the hypothesis that the great difference in inherited susceptibility to Friend virus between these 2 strains of mice is determined mainly at a single gene locus; that resistance and susceptibility to Friend virus are determined by two alleles (R and S, respectively, in the diagram); and that both alleles express themselves when they are in the heterozygous condition (codominance or no dominance over one another).

mainly, if not entirely, by a single allelic pair of genes. These are designated as R and S in text-figure 1.

No evidence of linkage was detected between inherited Friend virus susceptibility and sex. Nor was linkage found between Friend virus susceptibility and either of the coat color genes agouti or albino (*15*), which segregated in these crosses.

To find out whether the observed phenotypic ratios truly reflected underlying genotypes, male progeny of F_2 and backcross mice were mated, each to several SIM strain female mice, and the offspring, as young adults, were tested with Friend virus. Provided that the family size in each case was sufficiently large to provide significant data, the probable genotype of the animal being tested could be inferred from the types of response to Friend virus found in the progeny spleens. Thus if the spleens were all of F_1 hybrid (intermediate) type among the progeny of the cross between the male being tested and the SIM female (of presumed genotype SS), the conclusion would be warranted that the probable genotype of the male is RR. Similarly if the progeny were half of F_1 type and half of SIM type, then the genotype of the male would be considered to be RS, and if the progeny were all of SIM type, the genotype of the test male would be SS.

The genotypes of 20 F_2 male mice were tested in this way. Seventeen families gave unambiguous data. The genotypes distributed themselves into the three classes RR, RS, and SS, in the ratio 3:8:6, respectively ($\chi^2 = 1.12$; $0.3>P>0.2$). Of 14 SIM backcross mice tested, 13 families yielded useful data; the genotypes distributed themselves into 2 classes, RS and SS, in the ratio 7:6, respectively ($\chi^2 = 0.08$; $0.8>P>0.7$). Four of the F_2 males, whose genotypes had been tested by mating with SIM females, were also mated with C57BL/6 females, and the response of their progeny to Friend virus was determined. The genotypes deduced from the 2 different test crosses agreed with one another in each case.

These progeny-test results confirm the phenotypic data and establish the hypothesis that the inherited difference in susceptibility to Friend virus between SIM and C57BL/6 mice is determined predominantly, if not entirely, by a genetic difference at a single locus in these 2 strains.

In this conclusion, we concur with that reached by Odaka and Yamamoto (*7*) on the basis of crosses between strains RF and C57BL/6. However, our results cannot be reconciled with their view that Friend virus susceptibility is dominant over resistance.

In our experiments, F_1 hybrids produced by mating relatively insusceptible C57BL/6 mice with highly susceptible SIM were more susceptible than C57BL/6, but less so than SIM. This intermediate degree of susceptibility was also found in a proportion of F_2 mice and of the progeny of backcrosses to either parental strain, the ratios observed being consistent with those expected if both alleles at the locus controlling the susceptibility difference expressed themselves in the heterozygote. Susceptibility and resistance to Friend virus in this strain combination could thus be regarded as codominant traits or as traits exhibiting no dominance.

The discrepancy between the results of Odaka and Yamamoto and our own is not simply due to a difference in the strain combinations used in the two studies. We have examined the response of (C57BL/6 × RF)F_1 hybrids to graded doses of Friend virus with the spleen focus assay method, and have found their susceptibility to be intermediate between that of C57BL/6 and that of RF parental strain mice.

The lack of agreement between their results and our is probably due to the difference in methods used for determining host susceptibility to Friend virus. Their data were obtained by scoring mice as susceptible only after the animals had developed palpable splenomegaly. It would be difficult under such conditions to distinguish between animals of intermediate and those of high susceptibility, for the former could have gone on to develop the full-blown disease by the time they were scored.[7]

Our data would be consistent with the hypothesis that a single dose of the gene introduced by the C57BL parent would have the effect of reducing the number of Friend virus-induced foci that would ordinarily develop in the spleens of more susceptible mice; a double dose of this gene would reduce the number of spleen foci still further.

---

[7] *Note added in proof:*
Odaka and Yamamoto have recently also found the Friend virus susceptibility of F_1 hybrids to be intermediate between that of the susceptible (DDD) and that of the resistant (C57BL/6) parental strain, by using the spleen focus assay method (Jap J Exp Med 35: 311–314, 1965), and by comparing survival curves and incidence of splenomegaly after graded doses of the virus (*Ibid* 36: 23–31, 1966).

The mechanism of the C57BL gene-controlled effect on susceptibility to Friend virus is still unknown. Preliminary studies indicate that the early phases of infection in the relatively insusceptible C57BL strain are similar to those in the highly susceptible SIM strain. However, at later times, the quantity of virus recoverable from C57BL spleens is drastically reduced as compared with that from animals of the SIM strain (*16*). These results would suggest that animals possessing the R gene are thereby endowed with the capacity to interfere with the full development of the Friend virus infection *after* it has been initiated and even after it has progressed to a considerable extent. Further investigation of the process is in progress.

RELATION BETWEEN FRIEND VIRUS SUSCEPTIBILITY AND HISTOCOMPATIBILITY GENES IN MICE

Less than a year ago, Lilly, Boyse, and Old (*17*) reported evidence of an association between the histocompatibility-2 locus and inherited susceptibility to Gross leukemia virus in mice. They also indicated that a similar relation holds in the case of Friend virus. This intriguing report led us to look for differences in Friend virus susceptibility between congenic pairs of mice differing at single, known histocompatibility loci.

We have therefore been carrying out a survey of Snell's congenic resistant strains of mice (*18–22*)[8] and their inbred partners. Friend virus is injected intravenously into young adults of the respective strains, their spleens are scored as usual for macroscopic foci at 9 days, and the numbers compared.

Twenty-eight congenic strains and their 5 inbred partners have been examined to date. The survey covers the 9 histocompatibility loci so far isolated, from *H-1* to *H-11*, excluding the loci *H-5* and *H-6* (*23*) for which congenic lines are not available.

If Friend virus susceptibility were under the control of a histocompatibility gene (or a gene in the chromosome segment incorporated with it) then the degree of susceptibility of the congenic mouse should be related to that of the strain donating the foreign chromosome segment. Consequently, whenever these are still in existence, representatives of the donor strain are also examined for their susceptibility to Friend virus.

The inbred partner in all but 4 instances was C57BL/10ScSn, known to be highly resistant to induction of spleen foci by Friend virus (susceptibility index <0.001). What we were looking for in most experiments, then, was the presence of significantly increased numbers of spleen foci in groups of mice congenic with C57BL/10 and given large doses of Friend virus.

---

[8] In making this survey, we take our place among the many who are deeply indebted to Dr. G. D. Snell and his associates for having prepared and made available a large number of congenic resistant strains of mice.

Since the work is still in progress, all results must be regarded as preliminary, although they may be of sufficient interest to merit reporting at this stage.

No increase in focus number has been found to date in mice differing from C57BL/10 at *H-1*, *2*, *3*, *8*, *9*, *10*, or *11*. At *H-2*, seven strains congenic with C57BL/10 have been examined, each carrying a different *H*-allele. In no instance was evidence of increased susceptibility detected, despite the fact that in one case the chromosome segment bearing the foreign *H*-allele had been derived from the DBA/2 strain whose Friend virus susceptibility index was 3,000 times greater than that of C57BL/10. Nor was there any significant difference in Friend virus susceptibility between the congenic strains A.BY and A.CA, AKR.K and AKR.M, D1.LP and D1.C, and their inbred partners A/WySn, AKR/JSn, and DBA/1JSn, respectively, from which they were known to differ at *H-2*.

These observations neither refute nor support the evidence presented by Lilly (*24*) that a gene controlling susceptibility to Friend virus is linked to, but is situated at some distance from, the *H-2* locus. Such a gene would be expected to have become separated from *H-2* by crossing-over during the many generations which elapsed in the course of production of the congenic strains.

Preliminary evidence has been obtained which indicates that the susceptibility of strain B10.C(47N) mice to Friend virus is greater than that of C57BL/10ScSn mice; these strains differ at *H-7* (*21*), a locus not yet mapped. Suggestive, though still unconfirmed, evidence has also been found that a similar phenomenon may involve *H-4*. Results of these and further experiments in this system will be reported in detail later.

In conclusion, the results of the present study have thrown light on the mode of inheritance of susceptibility and resistance to the spleen focus-inducing action of Friend virus in 2 strains of mice, and they have shown that the spleen focus assay method is useful for genetic analysis.

REFERENCES

(*1*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307–318, 1957.

(*2*) Woolley, G. W., and Small, M. C.: Strain specificities of leukemia agent. Ann NY Acad Sci 68: 533–539, 1957.

(*3*) Rowe, W. P., and Brodsky, I.: A graded-response assay for the Friend leukemia virus. J Nat Cancer Inst 23: 1239–1248, 1959.

(*4*) Metcalf, D., Furth, J., and Buffett, R. F.: Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res 19: 52–58, 1959.

(*5*) Fieldsteel, A. H., Dawson, P. J., and Bostick, W. L.: Quantitative aspects of Friend leukemia virus in various murine hosts. Proc Soc Exp Biol Med 108: 826–829, 1961.

(*6*) Mirand, E. A., Prentice, T. C., Hoffman, J. G., and Grace, J. T.: Effect of Friend virus in Swiss and DBA/1 mice on Fe^{59}-uptake. Proc Soc Exp Biol Med 106: 423–426, 1961.

(*7*) Odaka, T. and Yamamoto, T.: Inheritance of susceptibility to Friend mouse leukemia virus. Jap J Exp Med 32: 405–413, 1962.

(*8*) Wahren, B.: Cytotoxic assays and other immunologic studies of leukemias induced by Friend virus. J Nat Cancer Inst 31: 411–423, 1963.

(*9*) Old, L. J., Boyse, E. A., and Lilly, F.: Formation of cytotoxic antibody against leukemias induced by Friend virus. Cancer Res 23: 1063–1068, 1963.

(*10*) Axelrad, A., and Steeves, R. A.: Assay for Friend leukemia virus: Rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology 24: 513–518, 1964.

(*11*) Pluznik, D. H., and Sachs, L.: Quantitation of a murine leukemia virus with a spleen colony assay. J Nat Cancer Inst 33: 535–546, 1964.

(*12*) Steeves, R. A., and Axelrad, A.: Application of a spleen focus assay method to quantitative immunological studies with Friend leukemia virus. Proc Amer Assoc Cancer Res 6: 61, 1965.

(*13*) Thomson, S., and Axelrad, A.: Sensitivity of Friend virus to UV and Co^{60} γ-radiation. Unpublished results.

(*14*) Axelrad, A.: Inheritance of susceptibility and resistance to Friend leukemia virus. In preparation.

(*15*) Gruneberg, H.: The Genetics of the Mouse, 2d ed. The Hague, Martinus Nijhoff, 1952.

(*16*) Axelrad, A., and Thomson, S.: Unpublished results.

(*17*) Lilly, F., Boyse, E. A., and Old, L. J.: Genetic basis of susceptibility to viral leukemogenesis. Lancet, 1964, pp 1207–1209.

(*18*) Snell, G. D.: Histocompatibility genes of the mouse. II. Production and analysis of isogenic resistant lines. J Nat Cancer Inst 21: 843–877, 1958.

(*19*) Snell, G. D., and Stevens, L. C.: Histocompatibility genes of mice. III. *H-1* and *H-4*, two histocompatibility loci in the first linkage group. Immunology 4: 366–379, 1961.

(*20*) Snell, G. D., and Bunker, H. P.: Histocompatibility genes of mice. IV. The position of *H-3* in the fifth linkage group. Transplantation 2: 743–751, 1964.

(*21*) ———: Histocompatibility genes of mice. V. Five new histocompatibility loci identified by congenic resistant lines on a C57BL/10 background. Transplantation 3: 235–252, 1965.

(*22*) Snell, G. D.: The production of coisogenic resistant lines of mice, and a list of available lines. *In* Methods in Medical Research (Eisen, H. N., ed.). Chicago, Year Book Med Publ, Inc., vol 10, 1964, pp 8–17.

(*23*) Amos, D. B., Zumpft, M., and Armstrong, P.: H–5.A and H–6.A, two mouse isoantigens on red cells and tissues detected serologically. Transplantation 1: 270–283, 1963.

(*24*) Lilly, F.: The histocompatibility-2 locus and susceptibility to tumor induction. Nat Cancer Inst Monogr 22: 631–642, 1966.

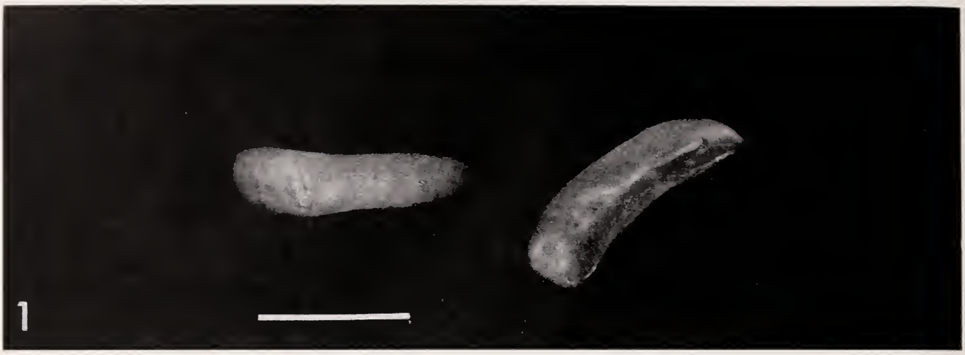
Spleens of mice 9 days after infection with a large dose of Friend virus. Bouin fixation. Scale marker = 1 cm.

FIGURE 1.—Spleens with no foci: C57BL type.

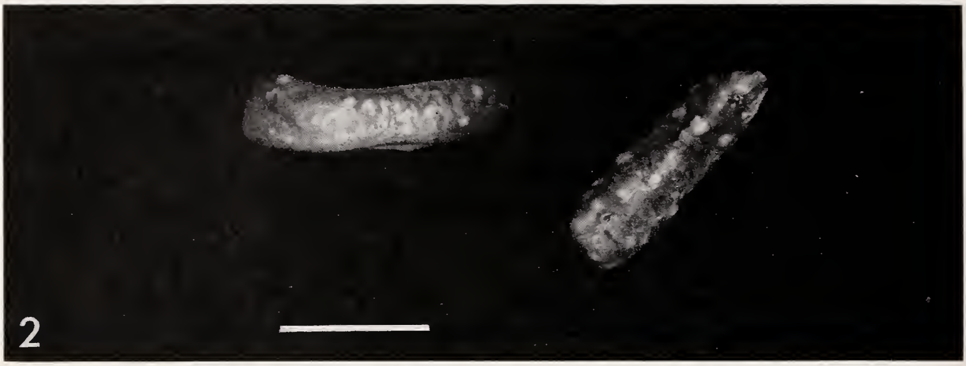
FIGURE 2.—Spleens with discrete, readily countable foci: F_1 hybrid or intermediate type.

FIGURE 3.—Greatly enlarged spleens with numerous confluent foci: SIM (Swiss inbred mouse) type.

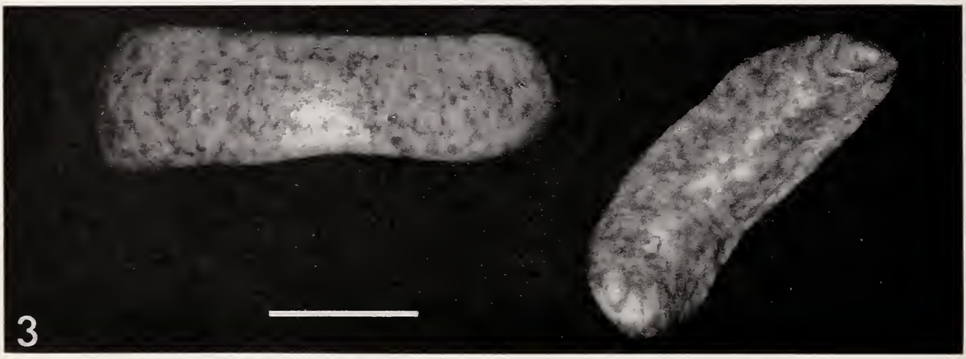
AXELRAD



1



2



3

DISCUSSION

Dr. Friend: I would like to congratulate Dr. Axelrad and his co-workers on a very excellent piece of work. We have been doing studies along this line, but using death from leukemia as the endpoint. We have essentially the same results, but after long periods of study. We have used the crosses between DBA/2, which are the most highly susceptible in our hands, and the C57BL/6 which are resistant.

One further word about the congenic strains. We tested DBA/1 mice congenic for the C57BL *H-2* locus, and these animals were just as susceptible as the original DBA/1 strain.

Dr. Axelrad: At *H-2*?

Dr. Friend: Right.

Dr. Axelrad: We have done the same thing and found it completely susceptible.

Dr. Latarjet: Did you take the sera of the animals on the 9th day and check the resistance associated with an increased immunological response against the virus?

Dr. Axelrad: Not yet. The data would look as if the most attractive hypothesis is an immunological one.

Dr. Breyere: I was wondering about resistance associated with loci 5 and 6. What were your results there? I'm not sure whether I missed them.

Dr. Axelrad: I said the 5 and 6 were named by Amos, not by Snell, and there are no congenic strains available at 5 and 6.

Dr. Lilly: I would like to say with respect to *H-5* and *H-6* that the Andervont and Bittner C3H substrains differ reciprocally at these loci. They are both equally susceptible to the Friend virus, which would lead one to suspect that *H-5* and *H-6* play no role in this susceptibility.

Chairman Rauscher: Arthur, if you pass virus serially in the very resistant strain BALB/c, does it adapt?

Dr. Axelrad: We have never passed the virus in BALB/c, but one of the things we want to look at is whether the strain in which the virus is cultivated has any influence on this.

The Histocompatibility-2 Locus and Susceptibility to Tumor Induction [1, 2]

FRANK LILLY,[3] *Sloan-Kettering Institute for Cancer Research, Sloan-Kettering Division, Cornell Graduate School of Medical Sciences, and Department of Genetics, Albert Einstein College of Medicine, New York, New York*

INVESTIGATIONS into the genetics of susceptibility to tumor induction in mice have generally revealed a type of multiple-factor inheritance. Malignancy appears after a threshold in the combined action of genetic and environmental factors has been attained (*1*).

The availability of many inbred mouse strains, differing from one another in their response to various tumorigenic agents, provides the materials with which one may hope to elucidate the nature of individual genetic influences on tumor susceptibility. Such individual influences have frequently been investigated by means of linkage studies wherein correlations are sought in segregating generations between known single-gene differences and susceptibility to tumorigenesis. In this manner, a gene influencing susceptibility to chemically induced lung tumors has been located in linkage group VII of the mouse (*2*, *3*). Thus, also, it has been found that marginal effects on susceptibility to mammary tumors are associated with alleles at the agouti (*4*), brown (*5*), and obese (*6*) loci. A relationship exists between the genes dilute (*7*) and flexed-tail (*8*), on the one hand, and susceptibility to leukemogenesis, on the other. The allele W^v of the dominant spotting gene appears to influence the development of ovarian adenomas (*9*).

In contrast to the multiple-factor determination of tumor susceptibility, studies of genetic factors in the susceptibility of mice to virus infection indicate that strain differences in susceptibility to individual viruses are generally due to one gene or, at most, to a small number of genes. The list

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by a grant from the American Cancer Society, Inc., and by Public Health Service contract PH 43-65-612 from the National Cancer Institute.

[3] *Present address:* Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461.

of viruses for which this has proved true includes yellow fever (*10*, *11*), ectromelia (*12*), mouse hepatitis (*13*), West Nile (*14*), Friend (*15*), polyoma (*16*), and influenza A (*17*).

Because of the relatively high degree of strain specificity shown by each of the murine leukemia viruses, it would seem *a priori* advantageous to investigate the genetic basis of susceptibility and resistance to these agents. Furthermore, the observation that the complex $H\text{-}2^k$ allele is a common feature of the genetic makeup of the four inbred mouse strains (AK, C58, C3H, and C57BR) most closely associated with susceptibility to the Gross leukemia virus made it appear that a determinant of susceptibility to this virus might be a gene lying within or very near the *H-2* (histocompatibility-2) locus. *H-2* is the major histocompatibility and blood group locus in the mouse genome.

This paper summarizes the results to date of our investigations of the relation between *H-2* and susceptibility to the Gross and Friend leukemia viruses, the Bittner mammary tumor virus, and the induction of skin tumors by 3-methylcholanthrene injected subcutaneously.

GROSS LEUKEMIA VIRUS

C3Hf/Bi mice ($H\text{-}2^k/H\text{-}2^k$) were rapidly and uniformly susceptible to the leukemogenic action of the Gross Passage A virus (*18*) inoculated neonatally (table 1). C57BL/6 mice ($H\text{-}2^b/H\text{-}2^b$), however, were relatively resistant to the virus, showing a 26% incidence of leukemia only after a much prolonged latent period. F_1 hybrid mice ($H\text{-}2^k/H\text{-}2^b$) of these two strains showed an even lower incidence of leukemia than the parental C57BL strain.

Two segregating populations derived from this cross were studied for susceptibility to Gross virus-induced leukemia: the (C3H × C57BL) × C3H backcross and the (C3H × C57BL)F_2 generation. The final incidences of leukemia in these populations as a whole were 72% in the backcross and 45% in the F_2 generation. Assuming that the number of independent genes (n) determining this susceptibility is related to the fraction

TABLE 1.—*H-2* type and Gross virus susceptibility

Strain or cross	*H-2* type	Leukemia incidence (%)		Mean latent period (days)
C3Hf/Bi	$H\text{-}2^k/H\text{-}2^k$	50/50	(100)	69
C57BL/6	$H\text{-}2^b/H\text{-}2^b$	32/123	(26)	191
(C3H × C57BL)F_1	$H\text{-}2^k/H\text{-}2^b$	11/126	(9)	258
C3H × (C3H × C57BL)	All types	163/224	(72)	87
	$H\text{-}2^k/H\text{-}2^k$	96/105	(91)	86
	$H\text{-}2^k/H\text{-}2^b$	67/119	(56)	90
(C3H × C57BL)F_2	All types	92/205	(45)	96
	$H\text{-}2^k/H\text{-}2^k$	54/58	(93)	94
	$H\text{-}2^k/H\text{-}2^b$	28/110	(25)	105
	$H\text{-}2^b/H\text{-}2^b$	10/37	(27)	82

of resistant animals (*rf*) in segregating populations by the following formulas

$$\text{backcross: } rf = 0.28 = (\tfrac{1}{2})^n, \text{ and}$$
$$F_2\text{: } rf = 0.55 = (\tfrac{3}{4})^n,$$

then, solving the equations for n, one finds that $n = 2$ is a solution with a high probability ($P > 0.21$ in the backcross and $P > 0.75$ in the F_2), whereas $n = 1$ and $n = 3$ are solutions with very low probabilities ($P < 0.0004$ in each case). From this, it appears that two independent recessive genes determine susceptibility to the Gross virus in the C3H substrain studied.

When the incidence of leukemia in these segregating populations is examined in relation to *H-2* type (table 1), it is seen that those mice homozygous for $H\text{-}2^k$ in both populations showed a >90% incidence of leukemia, whereas the other segregant classes showed a much lower incidence: 56% in the $H\text{-}2^k/H\text{-}2^b$ backcrosses and 25% for $H\text{-}2^k/H\text{-}2^b$ and 27% for $H\text{-}2^b/H\text{-}2^b$ in the F_2 generation. These data strongly indicate that one of the genes governing Gross virus susceptibility is located within or very near the *H-2* locus, while the other segregates independently.

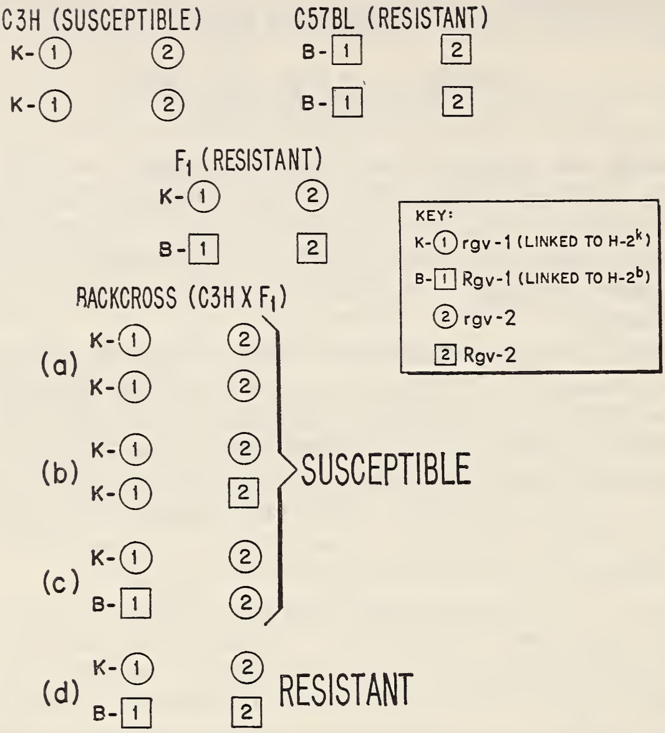
These two loci determining the response to Gross virus have been assigned the provisional symbols *Rgv-1* (linked to *H-2*) and *Rgv-2* (independent of *H-2*), and each has a dominant allele for resistance and a recessive allele for susceptibility to the virus. The theoretical relationship in the crosses studied between genotype at these two loci and at *H-2*, on the one hand, and phenotype for Gross virus susceptibility, on the other, is shown in text-figure 1. This scheme is consistent with the results obtained both in the backcross (illustrated) and in the F_2 generation (not illustrated).

It is not yet known whether *Rgv-1* is separable from the *H-2* locus. Moreover, the possibility that *Rgv-2* represents the combined effects of a number of factors cannot be excluded until this gene is located on the linkage map.

FRIEND LEUKEMIA VIRUS

Odaka and Yamamoto (*15*) reported that a single gene apparently determined susceptibility to the Friend virus (*19*) in the crosses they studied, but they did not locate the gene on the linkage map. We have conducted preliminary experiments to determine if this gene, like *Rgv-1*, might be associated with *H-2* (*20*).

Mice of the C3H/An strain ($H\text{-}2^k/H\text{-}2^k$) rapidly develop splenomegaly in response to inoculation with Friend virus, whereas mice of the I strain ($H\text{-}2^l/H\text{-}2^l$) are highly resistant to this effect of the virus. F_1 hybrids ($H\text{-}2^k/H\text{-}2^l$) are susceptible to the virus as newborns but quite resistant as adults. The (C3H × I) × C3H backcross inoculated with Friend virus


C3H (SUSCEPTIBLE)
C57BL (RESISTANT)
K-1
2
K-1
2
B-1
2
B-1
2
F1 (RESISTANT)
K-1
2
B-1
2
KEY:
K-1 rgv-1 (LINKED TO H-2k)
B-1 Rgv-1 (LINKED TO H-2b)
2 rgv-2
2 Rgv-2
BACKCROSS (C3H X F1)
(a)
K-1
2
K-1
2
(b)
K-1
2
K-1
2
(c)
K-1
2
B-1
2
SUSCEPTIBLE
(d)
K-1
2
B-1
2
RESISTANT

Text-figure 1.—Theoretical relationship between genotype at the *Rgv-1*, *Rgv-2*, and *H-2* loci and phenotype for Gross virus susceptibility. The possibility of recombination between the closely linked *Rgv-1* and *H-2* loci has been ignored.

as adults was examined for possible correlation between *H-2* type and Friend virus susceptibility.

The data from this experiment (table 2) are consistent with the single-gene hypothesis of Friend virus susceptibility and show that the two parental types (C3H: $H\text{-}2^k/H\text{-}2^k$, susceptible; F_1: $H\text{-}2^k/H\text{-}2^l$, resistant) have a significant tendency to segregate together. Thus, the gene determining susceptibility to the Friend virus appears to be linked to *H-2* with a recombination frequency of about 30%.

Similar experiments in the (C3H × I)F_2 generation (table 3) gave results compatible with the hypothesis of 30% recombination between *H-2* and the Friend virus susceptibility locus, though the proportion of

Table 2.—*H-2* type and Friend virus susceptibility in the (C3H/An × I) × C3H/An backcross

H-2 type	Susceptible	Resistant
$H\text{-}2^k/H\text{-}2^k$	36	16
$H\text{-}2^k/H\text{-}2^l$	14	34

TABLE 3.—*H-2* type and Friend virus susceptibility in the (C3H/An × I)F_2 generation

H-2 type	Virus dose	Susceptibility (%)	
$H\text{-}2^k/H\text{-}2^k$	0. 2	8/17	(47)
	0. 02	3/22	(14)
	0. 002	2/12	(17)
	Total	13/51	(25)
$H\text{-}2^k/H\text{-}2^l$	0. 2	8/39	(21)
	0. 02	1/34	(3)
	0. 002	0/21	(0)
	Total	9/94	(10)
$H\text{-}2^l/H\text{-}2^l$	0. 2	3/11	(27)
	0. 02	1/13	(8)
	0. 002	0/15	(0)
	Total	4/39	(10)
All types	0. 2	19/67	(28)
	0. 02	5/69	(7)
	0. 002	2/48	(4)
	Total	26/184	(14)

susceptible animals in the population corresponded to that expected on the basis of a single gene for virus susceptibility only in the group receiving the largest dose of virus.

These preliminary results are perhaps less conclusive than those obtained with the Gross virus. Several modifications of the system may be necessary for decisive results. For example, our criterion of Friend virus susceptibility was the development of splenomegaly, whereas a more promising one for future work appears to be the spleen focus tests (*21*). Also, it may be preferable to use an isogeneic source of virus; the virus used in these experiments was passed in a randombred strain of Swiss albino mice.

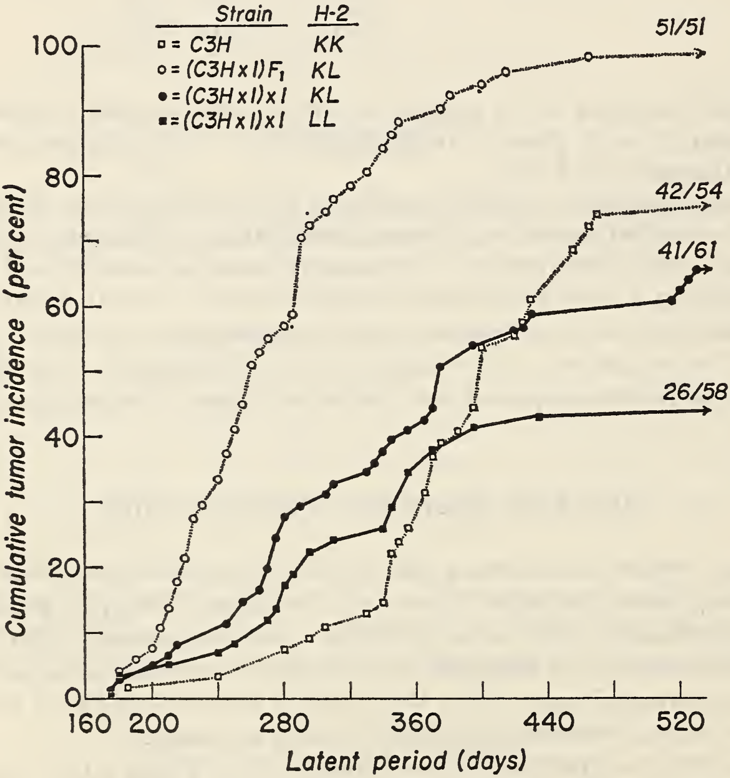
BITTNER MAMMARY TUMOR VIRUS

Many workers have stressed that at least three factors are involved in mammary tumor induction in mice: 1) the Bittner virus, 2) hormonal stimulation, and 3) genetic susceptibility. Since the patterns of hormone production seem to be inherited (*22*, *e.g.*), the only reason for separation of these hormonal factors from the "genetic" factors is that their mechanism of action is somewhat more clearly visible at present.

The C3H/An strain carries and transmits to its young a high level of active Bittner virus. Virgin females of this strain show a high incidence (>75%) of "spontaneous" mammary tumors, occurring mostly at 11 to 16 months of age.

The I strain, however, does not carry the Bittner virus, and its females show only a low incidence of mammary tumors even when the virus is introduced into them (*23*). Although genetically resistant to the virus, the I strain possesses an "accelerator factor," dominant in crosses with the C3H strain (*24*). Thus, whereas both C3H ($H\text{-}2^k/H\text{-}2^k$) and (C3H × I)$F_1$ ($H\text{-}2^k/H\text{-}2^l$) females have a high tumor incidence, the tumors appear about 4 months earlier in the hybrids than in the C3H females.

The cross studied for a possible correlation between *H-2* type and mammary tumor susceptibility was the (C3H × I) × I backcross, the hybrid mother being used to assure transmission of the virus to her litter. Text-figure 2 compares the cumulative tumor incidences in the two classes of *H-2* segregants of the backcross, in the parental C3H strain and in (C3H × I)F_1 females. The curves representing the two groups of *H-2* segregants do not appear significantly different from each other during the first year after birth. However, after about 400 days, the curve representing the cumulative tumor incidence in the $H\text{-}2^l/H\text{-}2^l$ segregants



TEXT-FIGURE 2.—Cumulative incidences of mammary tumors in Bittner virus-infected females of C3H/An, of (C3H/An × I) F_1, and of the $H\text{-}2^k/H\text{-}2^l$ and the $H\text{-}2^l/H\text{-}2^l$ segregant classes of the backcross (C3H/An × I) × I.

leveled off to a considerable extent, while that in the $H\text{-}2^k/H\text{-}2^l$ group continued to rise appreciably.

The tumor incidence in the $H\text{-}2^k/H\text{-}2^l$ segregants of this backcross attained 67%, whereas in the $H\text{-}2^l/H\text{-}2^l$ segregants the tumor incidence was 45%, a nonsignificant difference. When the experiment was terminated, the surviving animals were aged 17 to 21 months, and it is reasonable to expect that a few more late tumors would have arisen among them in the following months.

These results suggest inconclusively that a gene determining Bittner virus susceptibility may be associated with *H-2*. This hypothesis may be difficult to confirm, however, since so many incidental environmental factors can intervene during the prolonged observation period required.

3-METHYLCHOLANTHRENE

Because of the associations of *H-2* with susceptibility to these tumor viruses, it is particularly interesting to compare the results of a study concerning *H-2* in relation to tumor induction by a chemical substance.

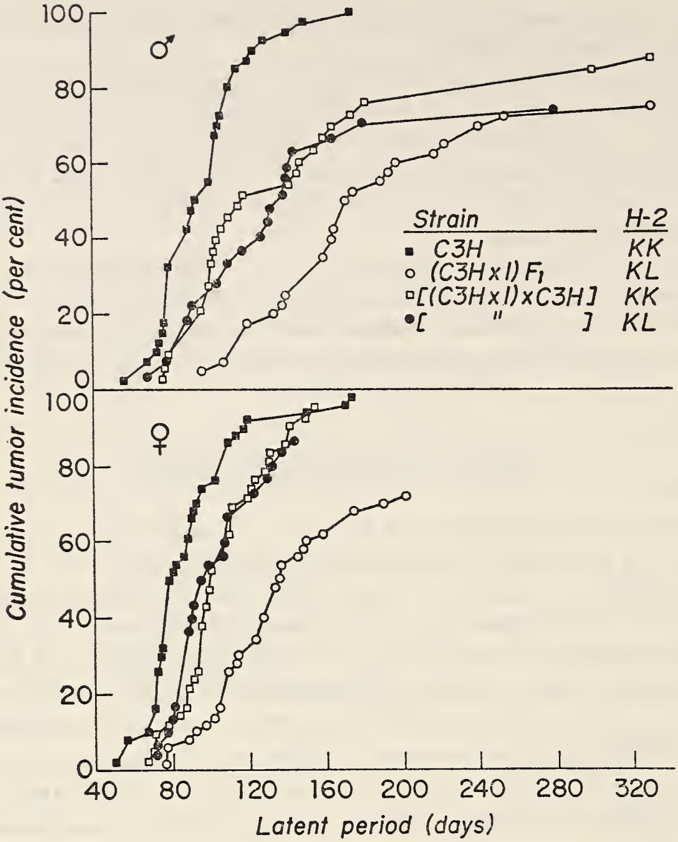
Burdette (*25*) observed the development of skin tumors induced by methylcholanthrene injection in strains C3H (susceptible) and JK (relatively resistant, with a considerably longer latent period for tumor induction), in their F_1 hybrids and in backcrosses to each parental strain. He found that the latent periods became longer and the final tumor incidence lower as the proportion of genetic material from the JK strain became larger in these populations. This implies a multiple-factor basis for the strain difference.

We have investigated the influence of *H-2* type on this susceptibility to 3-methylcholanthrene, using the susceptible C3H/An and the relatively resistant I strains, whose F_1 hybrids also show an intermediate response to methylcholanthrene injection (*26*).

Text-figure 3 shows the cumulative incidence of tumors of nonmammary origin among C3H ($H\text{-}2^k/H\text{-}2^k$), (C3H × I)$F_1$ ($H\text{-}2^k/H\text{-}2^l$), and the $H\text{-}2^k/H\text{-}2^k$ and $H\text{-}2^k/H\text{-}2^l$ segregant groups of the backcross generation between them. This chart indicates no detectable difference between the two classes of *H-2* segregants. Their incidence curves overlap throughout their length and are situated almost exactly midway between the curves for the C3H and the (C3H × I)F_1 groups. The final tumor incidences do not differ significantly from each other.

Thus there is no basis in this experiment for the conclusion that *H-2* plays any role in susceptibility to chemically induced skin tumors. Conceivably it may prove otherwise using another strain combination, a lower dose of carcinogen, or studying the other backcross combination, (C3H × I) × I.

MURINE LEUKEMIA



Text-figure 3.—Cumulative incidences of tumors of nonmammary origin induced by the subcutaneous injection of 3-methylcholanthrene in males and females of C3H/An, of (C3H/An × I)F_1, and of the $H\text{-}2^k/H\text{-}2^k$ and the $H\text{-}2^k/H\text{-}2^l$ segregant groups of the backcross (C3H/An × I) × C3H/An.

DISCUSSION

H-2 type in relation to susceptibility to four different oncogenic agents was studied in segregating generations of selected (susceptible × resistant) crosses.

Susceptibility to leukemogenesis by the Gross virus seems to be controlled by two independent genes in the crosses examined, and one of these (*Rgv-1*) is either identical with, or closely linked to, *H-2* in linkage group IX of the mouse, while the other (*Rgv-2*) segregates independently. Susceptibility to the Friend virus, however, is apparently due to a single gene linked to *H-2* with a recombination frequency of about 30%.

Preliminary and inconclusive evidence suggests that one of the genes determining resistance to mammary tumorigenesis by the Bittner virus in the I strain may be associated with *H-2*.

In contrast, there is no evidence that *H-2* plays any role in susceptibility to skin tumorigenesis after methylcholanthrene injection.

Thus, among the four oncogenic agents used in these studies, those seeming to have a possible association of susceptibility with *H-2* type were the three viruses, and of these, the best evidence of such an association was obtained with the two murine leukemia viruses.

Whether some part of the *H-2* locus itself is responsible for the positive effects or whether the genes detected are separate from *H-2* cannot yet be answered.

One reason for suspecting that it may indeed be *H-2* itself that governs susceptibility, especially with the Gross virus, is the association of the $H\text{-}2^k$ allele with this susceptibility in several mouse strains of independent origin. If the two loci (*i.e.*, *H-2* and *Rgv-1*) are not identical, then one would expect to find some strains bearing $H\text{-}2^k$ that were resistant to the virus, and this is not true for any $H\text{-}2^k$ strain tested with the Gross Passage A virus.

Nevertheless, at least two other genes closely associated with *H-2* merit attention as possible factors in the determination of Gross virus susceptibility. These are the *Ss* locus and the gene controlling the thymus leukemia (TL) antigen. The *Ss* gene (*27*), which is apparently within the *H-2* locus itself (*28*), determines the serum level of a specific globulin, and, among the inbred strains tested, only those possessing the $H\text{-}2^k$ allele were also of the *Ss-L* type (low serum concentration). This globulin might thus be some sort of inhibitor either of the virus or of leukemogenesis. But TL (*29*) is a surface isoantigen normally found only in the thymus—an organ of first importance in Gross virus leukemogenesis—where its presence is goverened by a gene very close to *H-2* (*30*). However, it is also found in many leukemias arising even in TL-negative strains. Still, since the strain distribution of TL correlates poorly with susceptibility to the Gross virus, its location near *H-2* and *Rgv-1* can only be taken as further evidence of the importance of this region of linkage group IX in leukemogenesis.

If the $H\text{-}2^k$ allele itself determines susceptibility to the Gross virus, then, since the *H-2* substance is associated with cell membrane surfaces, one possible hypothesis explaining the mechanism involved is that the $H\text{-}2^k$ substance might act as a receptor site for the virus, permitting its entry into and ultimate transformation of appropriate target cells. A major objection to this hypothesis is that Gross virus susceptibility is a genetically recessive trait, whereas *H-2* antigens are inherited codominantly. Thus, to account for the resistance of $H\text{-}2^k/H\text{-}2^b$ heterozygotes, one must postulate that a rather large patch of cell membrane occupied exclusively by the $H\text{-}2^k$ substance is necessary for successful entry of the virus into the cell, or that most molecules of the *H-2* substance are themselves $H\text{-}2^k/H\text{-}2^b$ molecular hybrids and that these are not functional virus receptors.

In any case, other genetically determined mechanisms of virus resistance are certainly not to be excluded from consideration. Several types of variants of genes influencing the capacity of the host to respond immunologically to the virus are easily conceivable, as are variants that alter the production of interferon. It is with the goal in mind of contributing to the elucidation of the mechanisms of susceptibility and resistance to the murine leukemia viruses that we are currently pursuing studies with these viruses.

SUMMARY

Susceptibility to the induction of tumors with four different oncogenic agents, the Friend and Gross leukemia viruses, the Bittner mammary tumor virus, and 3-methylcholanthrene, was studied relative to *H-2* type in segregating of generations of crosses of (susceptible × resistant) inbred mouse strains. Linkage was observed between *H-2* and susceptibility to the Gross and the Friend viruses and possibly to the Bittner virus. No linkage was detected between *H-2* and susceptibility to skin tumorigenesis by methylcholanthrene injection. These findings may provide a tool for the elucidation of genetic mechanisms in resistance to viral tumorigenesis.

REFERENCES

(*1*) Heston, W. E.: The genetic concept of the etiology of cancer. *In Proc* Fourth National Cancer Conf (1960), pp 73–82, 1961.

(*2*) ———: Effects of genes located on chromosomes III, V, VII, IX, and XIV on the occurrence of pulmonary tumors in the mouse. *In* Proc Internat Genetics Symposia (1956), Cytologica Suppl, pp 219–224, 1957.

(*3*) Bloom, J. L., and Falconer, D. S.: A gene with major effect on susceptibility to induced lung tumors in mice. J Nat Cancer Inst 33: 607–618, 1964.

(*4*) Heston, W. E., and Vlahakis, G.: Influence of the A^y gene on mammary-gland tumors, hepatomas, and normal growth in mice. J Nat Cancer Inst 26: 969–983, 1961.

(*5*) Bittner, J. J.: Inciting influences in the etiology of mammary cancer in mice. Research Conf on Cancer (1944), Washington, D.C., A.A.A.S., pp 63–96, 1945.

(*6*) Heston, W. E., and Vlahakis, G.: Genetic obesity and neoplasia. J Nat Cancer Inst 29: 197–209, 1962.

(*7*) MacDowell, E. C., Potter, J. S., and Taylor, M. J.: Mouse leukemia. XII. The role of genes in spontaneous cases. Cancer Res 5: 65–83, 1945.

(*8*) Law, L. W.: The flexed-tail-anemia gene (*f*) and induced leukemia in mice. J Nat Cancer Inst 12: 1119–1126, 1952.

(*9*) Russell, E. S., and Fekete, E.: Analysis of *W*-series pleiotropism in the mouse: Effect of W^vW^v substitution on definitive germ cells and on ovarian tumorigenesis. J Nat Cancer Inst 21: 365–381, 1958.

(*10*) Sabin, A. B.: Genetic, hormonal and age factors in natural resistance to certain viruses. Ann NY Acad Sci 54: 936–944, 1952.

(*11*) ———: Nature of inherited resistance to viruses affecting the nervous system. Proc Nat Acad Sci 38: 540–546, 1952.

(*12*) Schell, K.: Studies on innate resistance of mice to infection with mousepox. II. Route of inoculation and resistance; and some observations on the inheritance of resistance. Aust J Exp Biol Med Sci 38: 289–300, 1960.

(*13*) Bang, F. B., and Warwick, A.: Mouse macrophages as host cells for the mouse hepatitis virus and the genetic basis of their susceptibility. Proc Nat Acad Sci 46: 1065–1075, 1960.

(*14*) Goodman, G. T., and Koprowski, H.: Macrophages as a cellular expression of inherited natural resistance. Proc Nat Acad Sci USA 48: 160–165, 1962.

(*15*) Odaka, T., and Yamamoto, T.: Inheritance of susceptibility to Friend mouse leukemia virus. Jap J Exp Med 32: 405–413, 1962.

(*16*) Chang, S.-S., and Hildemann, W. H.: Inheritance of susceptibility to polyoma virus in mice. J Nat Cancer Inst 33: 303–313, 1964.

(*17*) Lindemann, J.: Inheritance of resistance to influenza virus in mice. Proc Soc Exp Biol Med 116: 506–509, 1964.

(*18*) Gross, L.: Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proc Soc Exp Biol Med 94: 767–771, 1957.

(*19*) Friend, C.: Cell-free transmission in adult Swiss mice of a disease having the character of leukemia. J Exp Med 105: 307–318, 1957.

(*20*) Lilly, F., Boyse, E. A., and Old, L. J.: Genetic basis of susceptibility to viral leukaemogenesis. Lancet 2: 1207–1209, 1964.

(*21*) Axelrad, A. A., and Steeves, R. A.: Assay for Friend leukemia virus: Rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology 24: 514–518, 1964.

(*22*) Badr, F. M., and Spickett, S. G.: Genetic variation in the biosynthesis of corticosteroids in *Mus musculus*. Nature (London) 205: 1088–1090, 1965.

(*23*) Andervont, H. B.: Fate of the C3H mammary tumor agent in mice of strains C57BL, I, and BALB/c. J Nat Cancer Inst 32: 1189–1198, 1964.

(*24*)———: The incidence of induced subcutaneous and spontaneous mamary tumors in hybrid mice. Public Health Rep 53: 1665–1671, 1938.

(*25*) Burdette, W. J.: The inheritance of susceptibility to tumors induced in mice. III. Tumors induced with methylcholanthrene in the backcross of C3H and JK mice. J Nat Cancer Inst 9: 105–110, 1948.

(*26*) Old, L. J., Boyse, E. A., Clarke, D. A., and Carswell, E. A.: Antigenic properties of chemically induced tumors. Ann NY Acad Sci 101: 80–106, 1962.

(*27*) Shreffler, D. C., and Owen, R. D.: A serologically detected variant in mouse serum: Inheritance and asssociation with the histocompatibility-2 locus. Genetics 48: 9–25, 1963.

(*28*) Shreffler, D. C.: A serologically detected variant in mouse serum: Further evidence for genetic control by the histocompatibility-2 locus. Genetics 49: 973–978, 1964.

(*29*) Old, L. J., Boyse, E. A., and Stockert, E.: Antigenic properties of experimental leukemias. I. Serological studies *in vitro* with spontaneous and radiation-induced leukemias. J Nat Cancer Inst 31: 977–986, 1963.

(*30*) Boyse, E. A., Old, L. J., and Luell, S.: Genetic determination of the TL (thymus leukemia) antigen in the mouse. Nature (London) 201: 779, 1964.

DISCUSSION

Dr. Tennant: My, he has been busy—both physically and mentally. This is a fascinating paper and a great deal of data has been presented. I am, of course, glad to see that others are finding an influence for genetic factors, particularly histocompatibility factors, on susceptibility to various types of virus-induced leukemia and tumors.

I wish to ask Dr. Lilly a question about his reference, following Dr. Axelrad's paper, to the C3H/Bi and C3H/An difference being at the *H-5* and *H-6* loci. In looking for a reference defining *H*-gene differences among C3H sublines, I finally found one by Amos where there was quite a lot of careful work done in differentiating substrains of C3H, but I saw no specific reference to C3H/Bi or C3H/An, although there was an NIH strain listed, as I recall.

Before he answers the question, I just want to say that this is very interesting in the light of Dr. George Woolley's results of a good many years ago, soon after he had confirmed Dr. Gross' work. He did studies on subline intrastrain susceptibilities, and found a striking differenc between these two sublines of C3H, *Bi* and *An*. Dr. Bittner at that time was at Minnesota and I asked him about it. He said these two lines had been separated for 30-odd years and had not taken one another's mammary tumor transplants for years, which one would not expect, if the histocompatibility difference were as minor as *H-5* and *H-6*. Are you certain these were the sublines? If so, I'd like the reference later.

Dr. Lilly: Yes, I am sure. We have worked considerably with both the Bittner and Andervont substrains and these are quite different. I don't know whether they differ additionally at other histocompatibility loci—it's very possible—but Amos' work originally involved the Heston and the Strong sublines of the C3H which are respectively equivalent to the Andervont and the Bittner. This is terribly confusing; there are too many available substrains of C3H.

These differ reciprocally at *H-5* and at *H-6*, so it is at least a two-gene difference there, which may explain why—or may not—they don't take grafts of mammary tumors. They do not accept skin grafts; that we've tried. That was obivous long ago.

I have one comment about what you said of Dr. Gross' early work in which he found that, at that stage, working with much lower potency virus preparations, before he developed the Passage A virus, there were C3H substrain differences in susceptibility to his virus.

We have given a good bit of thought to that and we have a hypothesis which we haven't yet resolved. Is it not possible that the relatively susceptible strain at that point possessed both of the genes for susceptibility *rgv-1* and *rgv-2*, whereas those which were resistant possessed only one? This, again, is a hypothesis. On the other hand, you may be correct in that there is some influence by *H-5*. We have specifically tested *H-6* which showed no correlation with susceptibility in the crosses studied.

Dr. Tennant: Thank you. Regarding your reference to the low globulin fraction in possible association with susceptibility and $H\text{-}2^k$ linkage. I just wanted to take exception to your saying "susceptibility to viral leukemogenesis," *per se*. Such an effect would really be specific to the virus, I would think, since there appears to be an inverse relationship, depending on the virus source. In other words, the $H\text{-}2^k$ group is very susceptible to the virus you have been using—very resistant to the virus I have been working with.

Response of Rauscher Virus to Drug and Immune Serum Therapy *In Vivo*[1]

Michael A. Chirigos,[2] Estelle D. Luber,[2] Srikrishna Vadlamudi,[3] *and* Mary A. Fink,[4] *National Cancer Institute, and Microbiological Association, Inc., Bethesda, Maryland*

The viral etiology of lymphocytic leukemia in a specific inbred strain of mice was first demonstrated by Gross (*1*) in 1951. Since this pioneer study, development of the murine leukemia field has been so rapid that there are now at least 14 distinct viruses known to induce leukemia in rodents. The literature pertaining to these viruses has been reviewed by Gross (*2*), Sinkovics (*3*), and more recently by Moloney (*4*, *5*).

In addition to the murine leukemias, there are the oncogenic viruses which are infectious for chickens (Rous sarcoma, avian myeloblastic and erythroblastic leukemias, and avian lymphomatosis), rabbits (myxoma, fibroma, and papilloma), and hamsters and rats (polyoma). The Bittner agent has been reported to produce carcinomas in mice (*6*). Human adenoviruses have been shown to be oncogenic in experimental animals (*7*, *8*).

To prove that oncogenic viruses have a possible role as causative agents in the development of human tumors is a most difficult task. Virus particles have been shown in the Burkitt lymphoma (*9*) and human myeloid leukemia cells (*10*). If viruses comparable to those now known to be associated with avian and murine leukemia are etiological agents of human leukemia, an effective means for their control is necessary.

It is a generally held belief that at present there are no agents known to be capable of exerting a real chemotherapeutic action in human viral infection. This, however, is not strictly true, since two classes of compounds have been demonstrated to be effective against naturally occurring specific virus infections in man: the thiosemicarbazones against smallpox (*11*) and 5-iodo-2′-deoxyuridine against herpes simplex keratitis (*12*). Adamantanamine appears to be a possible chemoprophylactic agent for influenza infection (*13*).

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Drug Evaluation Branch, Cancer Chemotherapy National Service Center, and Laboratory of Viral Oncology, National Cancer Institute, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Md.

[3] Microbiological Associates, Inc., Bethesda, Md. Contract No. PH-43-64-911.

[4] The authors are grateful to Mr. H. Pettigrew of the Biometry Branch, National Cancer Institute, for advice and assistance in the statistical analysis.

Approaches to the control of viral infection, other than chemotherapy, are immunological, and possibly interferon prophylaxis. However, to justify application of any of these therapeutic approaches to man, *in vivo* trials with naturally occurring viral diseases in lower animals must be made. Because of several similarities between virus-induced animal leukemia and human leukemia, a murine virus-induced leukemia test system would appear to be a logical choice for evaluating antiviral agents. The Friend (*14–16*), Rauscher (*17–19*), and Moloney (*20*) murine leukemogenic viruses have been employed for testing potential antiviral agents.

In this communication we report the use of an *in vivo* method, utilizing the Rauscher leukemogenic virus (*19*), for evaluating the antiviral effectiveness of drug- and immuno-therapy.

MATERIALS AND METHODS

Virus inoculum.—The preparation of standard lots of Rauscher virus has been reported previously (*21*). For each experiment, a frozen aliquot of virus was allowed to thaw and was diluted to a $10^{-1.3}$ concentration in sterile 0.9% NaCl. A volume of 0.2 ml was inoculated intraperitoneally into 6- to 8-week-old BALB/c mice[5] weighing between 21 and 25 g.

Randomization of test animals.—In all experiments mice were randomized shortly after inoculation and were then distributed into appropriate groups.

Drug and treatment.—6-Mercaptopurine (6-MP) was dissolved in dilute alkali and administered subcutaneously in the axillary region in a constant volume of 0.01 ml per g of body weight. The number of treatments is shown with each experiment.

Rauscher virus immune serum.—Rauscher virus antibody, made in BALB/c mice, was obtained from Dr. M. A. Fink, National Cancer Institute. The method for preparing neutralizing antibody for Rauscher virus has been described by Fink (*22*). In the immunotherapy experiments, mice were injected intraperitoneally with 0.25 or 0.5 ml of homologous normal or immune serum. The number of treatments is shown with each experiment.

Scheme for in vivo antiviral assay.—The scheme employed for the study of the influence of drug- or immuno-therapy on the virus and virus-host relationship was as follows: Mice inoculated intraperitoneally with virus were divided into control and treated groups and served as donor mice for bioassay. In each experiment therapy was initiated when mice were viremic; this time is shown with each experiment. On specified days, individual virus-inoculated control and treated donor mice were anesthetized with ether and bled by severing the brachial artery. Spleen weights of these mice were recorded. Blood was separated by centrifugation (2150 × g) for 5 minutes. Serial tenfold dilutions of plasma were immediately prepared in sterile 0.9% NaCl, and 0.2 ml was inoculated intraperitoneally into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed by cervical fracture and their spleens were weighed individually on a model P120 Mettler balance. Spleen weights were read to the nearest 10 mg.

---

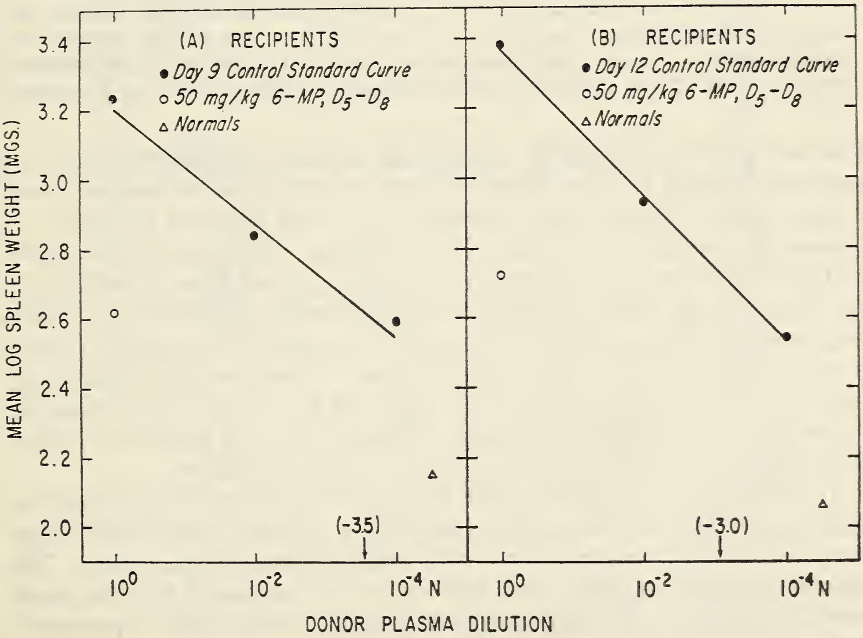
[5] Obtained from the National Institutes of Health Breeding Colony.

RESULTS

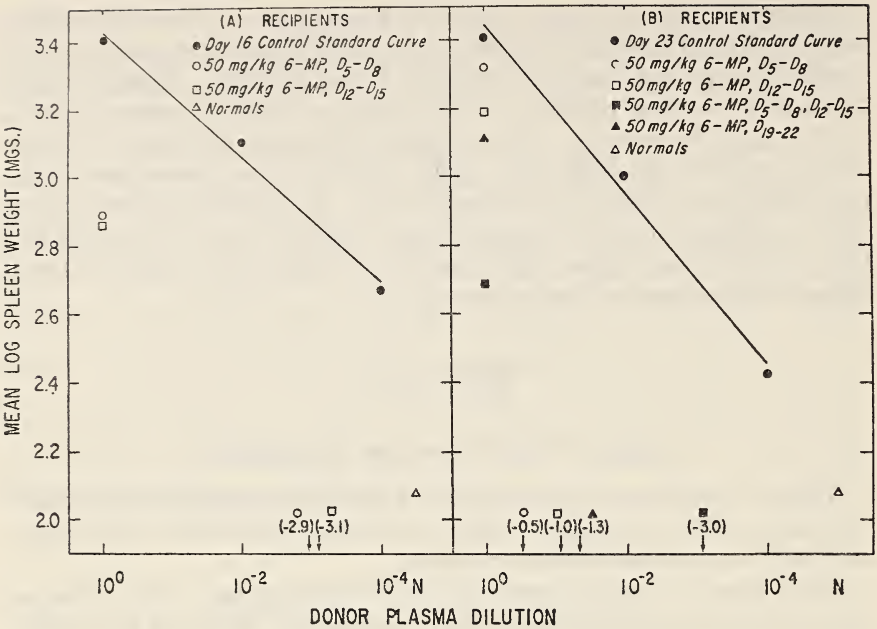
Effect of 6-MP Therapy on Viremia

Different schedules of treatment with 6-MP were employed to determine the length of time its antiviral effect was demonstrable and to what extent the drug treatment reduced host viremia.

Text-figures 1A and 1B and 2A and 2B present the results obtained when viremic animals received different regimens of treatment with 6-MP.



TEXT-FIGURE 1A AND 1B.—Effect of 6-mercaptopurine treatment on virus recovery. Virus-infected control and treated donor animals killed on the 9th and 12th day after virus inoculation. Pooled plasma from 5 donor mice was bioassayed into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed for spleen-weight determination. Means based on 5 animals.



TEXT-FIGURE 2A AND 2B.—Effect of 6-mercaptopurine treatment on virus recovery. Virus-infected control and treated donor animals killed on the 16th and 23d day after virus inoculation. Pooled plasma from 5 donor mice was bioassayed into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed for spleen-weight determination. Means based on 5 animals.

The straight lines (fitted by the method of least squares) represent the response observed in recipient animals given inoculations of control donor plasma and facilitate over-all comparison of the effect of treatment on viremia in treated and untreated donor animals. Standard curves, representing the amount of recoverable virus from control donor mice killed 9, 12, 16, and 23 days after infection, were based on the results obtained when recipient mice subinoculated with serial dilutions of control donor plasma were killed for spleen-weight determinations. The amount of virus (log) recovered from treated animals was estimated by comparing the mean log spleen weight of recipient animals, subinoculated with undiluted plasma from drug-treated donors, to the appropriate standard curve.

To determine the length of time after infection necessary to attain the maximum virus titer, virus-inoculated donor animals were killed at successive times and the plasma was subinoculated into recipient mice. The results presented in table 1 and text-figure 3, as indicated by the splenomegalic response achieved in recipient mice, show that virus is present in plasma in relatively large amounts as early as 3 days after infection, and that the level of virus increases rapidly until about the 12th day when a plateau is reached. These observations are in agreement with results reported by Rauscher (*23*).

TABLE 1.—Summary of 6-mercaptopurine (6-MP) antiviral activity

Treatment 6-MP (50 mg/kg)	Difference in days*	Day of bioassay	Donor spleen weight (mg)†	Recipient spleen weight‡ (mg)†	Normal spleen weight (mg)†	Difference§ in recoverable virus (log)	MST‖ (days)
				10^0			
—		5	268	1282		—	
—		9	474	1770	108	—	
D5–8	1	9	188	420		3.5	
—		12	592	2454	117	—	
D5–8	4	12	202	534		3.0	
—		16	1166	2590	122	—	
D5–8	8	16	308	794		2.9	
D12–15	1	16	292	728		3.0	
—		23	2326	2598	115	—	74
D5–8	15	23	690	2134		0.5	138
D12–15	8	23	534	1552		1.0	147
D5–8 D12–15	8	23	290	492		3.0	115
D19–22	1	23	508	1328		1.3	98

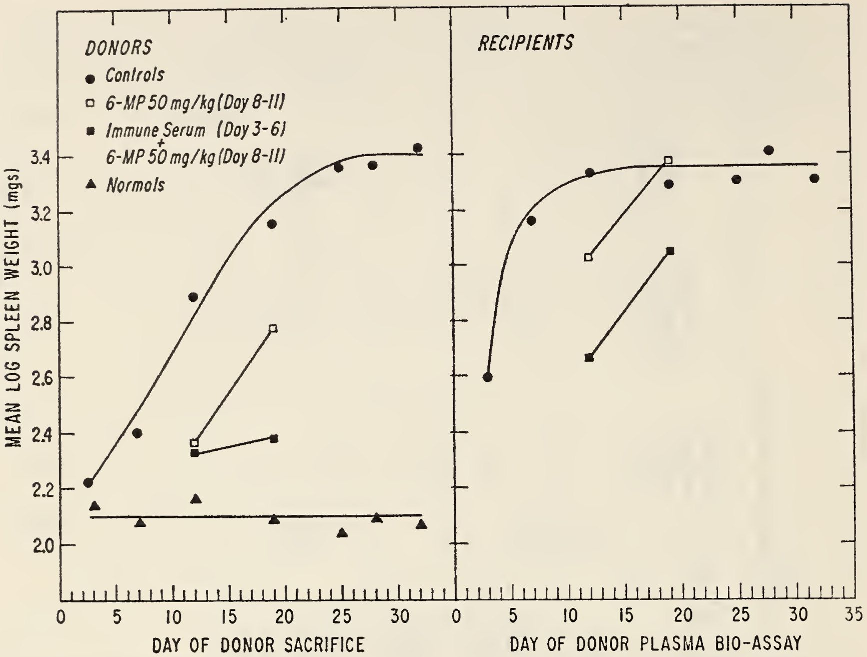
*Difference in time (days) from last treatment and day of bioassay.

†Average of 5 animals.

‡Recipient mice inoculated with undiluted donor plasma (10^0) and killed 21 days later.

§Difference in amount of recoverable virus (log) estimated from control standard curve.

‖MST = median survival time.



Text-figure 3.—Effect of 6-mercaptopurine and combined 6-mercaptopurine and immune serum treatment on splenomegaly and viremia. Undiluted pooled plasma from 5 donor mice, killed on successive days after virus inoculation, was bioassayed into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed for spleen-weight determination. Donor and recipient means based on 5 animals.

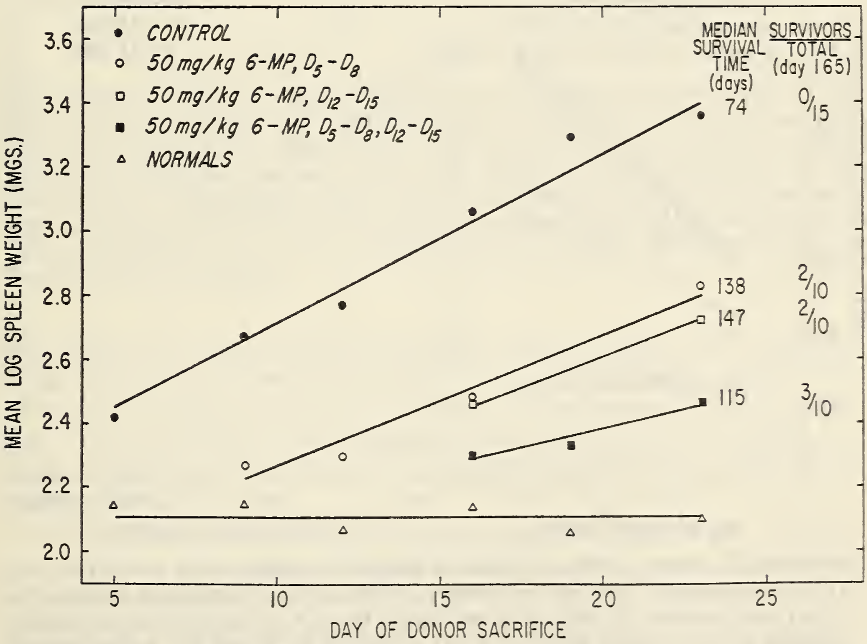
A single early course of 6-MP therapy (D5-8) markedly reduces recoverable virus at first; however, the virus titer then rises rapidly to a point at which it approaches that of the untreated controls. This is illustrated in text-figures 1A and B and 2A and B, where it can be seen that 1, 4, 8, and 15 days following cessation of therapy there was a 3.5, 3.0, 2.9, and 0.5 log reduction in virus titer, respectively. This diminishing difference in virus titer between the treated and control animals indicates that this regimen of treatment is effective in suppressing viral replication for only 8 days. The result of treatment from day 12 through day 15 was a 3.1 log reduction in recoverable virus (text-fig. 2A). Eight days after the last treatment, a 1.0 log difference in recoverable virus was still evident (text-fig. 2B). Treatment from day 19 through day 22 reduced the virus titer only by 1.3 logs. A comparison of the antiviral activity achieved by the different regimens of therapy tested (text-fig. 2B) shows that the most sustained antiviral effect was obtained by sequential therapy (D5-8, D12-15).

Effect of 6-MP Therapy on Splenomegaly and Survival Time

In addition to studying the effect of drug therapy on host viremia, its effect on splenomegaly and host survival was examined (text-fig. 4, table 1). The straight lines (fitted by the method of least squares), shown in text-figure 4, facilitate a comparison of the increase in spleen weight of treated and control animals. The control line shows that the weight of spleens from infected animals increased at a fairly constant rate from the 5th to the 23d day, at which time they weighed approximately 2.3 g (table 1). This represents a 20-fold increase in weight over that of spleens from normal uninoculated mice killed at the same time.

Each regimen of drug treatment tested resulted in a reduction of donor spleen weight (text-fig. 4). It is interesting that the retardation of splenomegaly achieved by the three regimens of treatment persisted over the 23-day observation period. Sequential therapy resulted in the most marked and persistent reduction in spleen weight.

Substantial increases in survival time were also achieved with therapy (table 1, text-fig. 4). Increases in survival times of 86, 98, 55, and 32% over untreated controls were attained by treatment on days 5 through 8, days 12 through 15, days 5 through 8 and days 12 through 15, and days 19 through 22, respectively. All the drug-treated mice surviving for 165



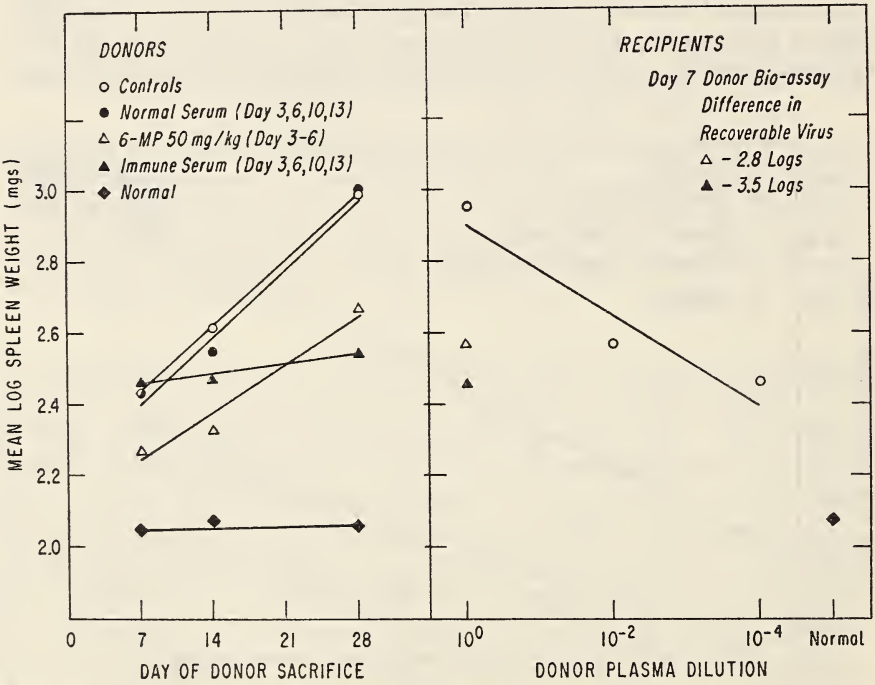
Text-figure 4.—Effect of different regimens of 6-mercaptopurine treatment on splenomegaly and survival time of virus-infected animals. Means based on 5 animals.

days (text-fig. 4) showed signs of the virus-induced disease, as evidenced grossly by enlarged spleens.

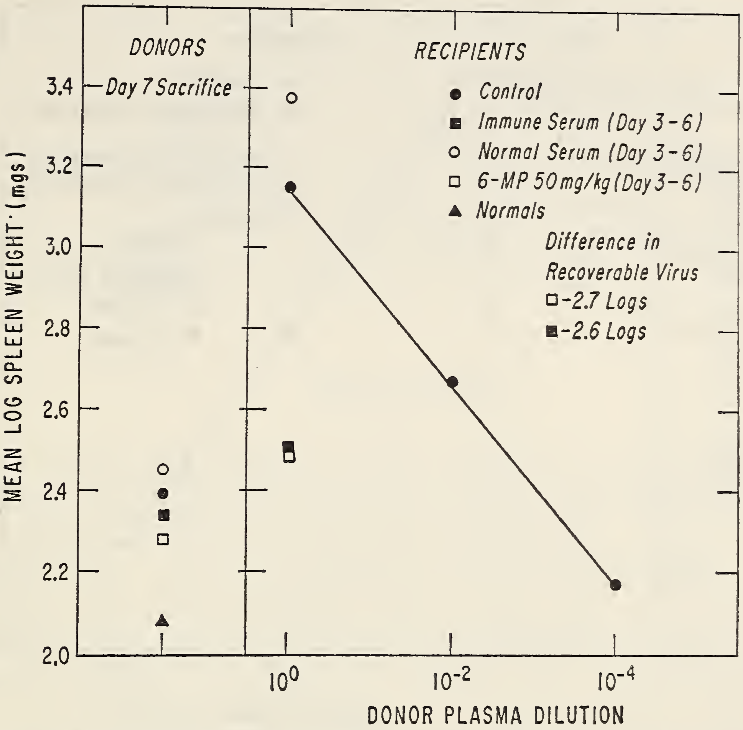
Effect of 6-MP and Immune Serum Therapy on Splenomegaly and Viremia

It has been reported that mice are protected against Rauscher virus infection by active immunization (*22*, *24*); therefore, it was desirous to determine whether combined treatment with drug and immune serum would be beneficial. The results of treating Rauscher virus-infected mice with drug and immune serum are presented in text-figures 3, 5, 6, and 7.

In the first experiment (text-fig. 5), infected donors were killed at successive times to evaluate the effect that treatment exerted on splenomegaly. The spleen weight of untreated controls increased at a fairly constant rate from the 7th to the 28th day. Treatment with normal mouse serum did not affect the rate of splenomegaly. Drug and immune serum treatment



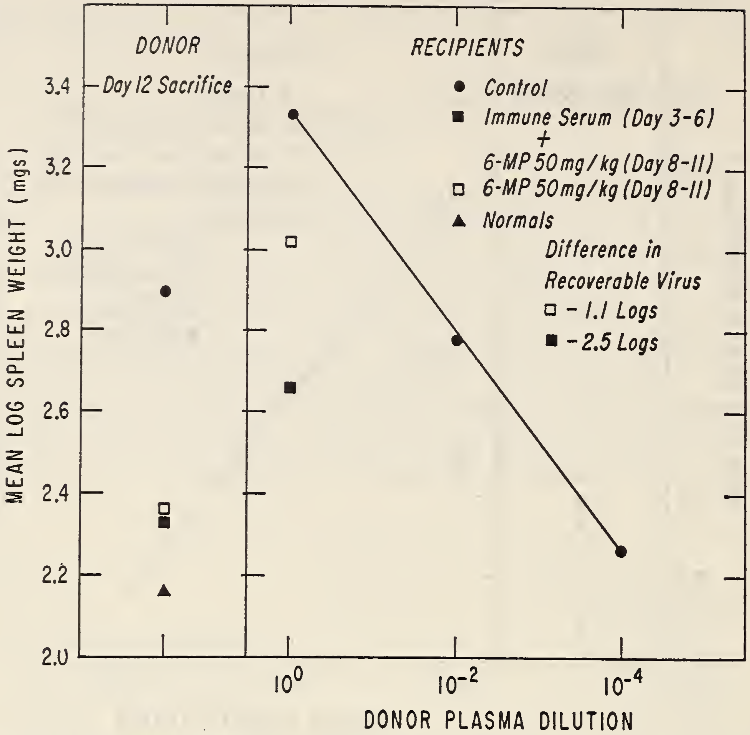
TEXT-FIGURE 5.—Effect of 6-mercaptopurine and normal and immune serum (Lot No. 1) on splenomegaly and virus recovery. 6-Mercaptopurine-treated animals received one injection of drug daily on days 3 through 6. Serum-treated animals received one injection of serum (0.5 ml) on days 3, 6, 10, and 13. Pooled plasma from 5 donor mice, killed 7 days after virus inoculation, was bioassayed into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed for spleen-weight determination. Donor and recipient means based on 5 animals.



TEXT-FIGURE 6.—Effect of 6-mercaptopurine and normal and immune serum (Pool No. A) on splenomegaly and virus recovery. 6-Mercaptopurine-treated animals received one injection of drug daily on days 3 through 6. Serum-treated animals received one injection of serum (0.25 ml) daily on days 3 through 6. Pooled plasma from 5 donor mice, killed 7 days after virus inoculation, was bioassayed into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed for spleen-weight determination. Donor and recipient means based on 5 animals.

each produced a substantial retardation in splenomegaly, but it appeared that immune serum treatment resulted in a more persistent effect. The virus titers of donors killed 7 days after infection were estimated by bioassay in recipient mice. Two treatments with immune serum resulted in a better reduction in virus titer than was achieved with four treatments of 6-MP (text-fig. 5).

Combination treatment was attempted in a second experiment (text-figs. 3, 6, and 7). Treatment with either 6-MP or immune serum alone resulted in a decrease of spleen weight and virus titer in donors killed 1 day after the last treatment (text-fig. 6). These results agree with the previous findings obtained in the first experiment (text-fig. 5). Normal serum treatment was again shown to be ineffective. The results shown in text-figure 7 represent the response observed in mice receiving combined drug and immune serum therapy. Spleen weights of donor mice treated with



TEXT-FIGURE 7.—Effect of 6-mercaptopurine (6-MP) and combined 6-MP and immune serum treatment on splenomegaly and virus recovery. Serum-treated animals received one injection of immune serum (0.25 ml) daily on days 3 through 6. 6-MP treatment initiated 8 days after virus inoculation and administered once daily on days 8 through 11. Pooled plasma from 5 donor mice, killed 12 days after virus inoculation, was bioassayed into groups of recipient mice. At 21 days post inoculation of donor plasma, the recipient mice were killed for spleen-weight determination. Donor and recipient means based on 5 animals.

6-MP or the combination of 6-MP and immune serum were markedly reduced, but a greater reduction of virus titer was achieved by combined therapy. Thus, combined therapy was shown to be as effective as a second course of treatment with 6-MP (text-fig. 2B). The availability of larger amounts of immune serum will allow further studies to determine whether enhanced therapeutic efficacy can be achieved by combined drug and immunotherapy. The results attained in this study are, at the least, suggestive.

Text-figure 3 summarizes the results of combined immune serum and drug therapy. The untreated donor control curve shows that the weight of spleens from infected animals increased at a fairly constant rate from the 3d to the 25th day, with a slight plateau region developing after the 25th day. Both treatment with 6-MP alone and the combined treatment resulted in a reduction of spleen weight that persisted over the 19-day observation period. However, the combined therapy resulted in a more marked and persistent reduction in spleen weight.

The course of viremia in treated and untreated animals was estimated by bioassaying undiluted plasma from donors killed at successive times into recipients killed 21 days after infection for spleen weights. Maximum virus titer in untreated controls was reached by the 12th day after infection. Virus titer in drug-treated animals was reduced at first; however, 8 days after the last treatment, the virus titer was similar to that in untreated controls. The combined treatment resulted in a more marked and persistent reduction of virus titer.

DISCUSSION

The persistently low amount of virus recoverable from plasma of mice treated early (D5-D8) reflects that therapy is more effective during the early phase of viremia when virus titer is relatively low. However, without subsequent treatment, virus replication progresses and virus titer increases rapidly. When a second course of treatment was initiated on the 12th day, virus titer was still low; thus the effect of the second course of treatment was to sustain the decrease in virus titer for a longer period. This sequential regimen of therapy was also effective in retarding splenomegaly. In view of the effective reduction in virus titer and spleen weight observed in the animals receiving sequential therapy, it would seem that a greater than 55% increase in survival time should have been achieved. No explanation can be presented for this result. The possibility of cumulative drug toxicity was ruled out, since no appreciable body weight loss occurred as a result of the regimen of treatment. It is interesting, however, that 30% of the animals in this group were surviving when the experiment was terminated. Treatment initiated at a time when viremia was maximal resulted in only a transitory decrease of virus titer. The lower degree of antiviral activity attained by delaying therapy until the 19th day may be attributable to the inability of this dose of drug treatment to effectively suppress the potent host viremia and induced leukemia at this time. In previous studies (*17*, *18*, *21*) histological examination of tissues revealed that, by the 17th day after virus infection, pathological changes progressing from hyperplasia and erythrocytopoiesis to generalized lymphatic leukemia had occurred.

The results of this study illustrate the sequence of events that ultimately lead to death of the virus-infected host, *i.e.*, viremia, splenomegaly, and leukemia. It appears that, as a consequence of drug therapy, host viremia was decreased, splenomegaly was retarded, and animals survived for a longer period than untreated controls. The ability of 6-MP treatment to decrease virus titer and increase survival time of Rauscher-infected animals confirms results previously reported (*17–19*). More extensive and detailed studies would be helpful in understanding how these changes were accomplished. Examination of tail blood smears from mice 38 days after Rauscher virus infection revealed the presence of an appreciable number of lymphoblasts, bands, myelocytes, and metamyelocytes (*18*). It would be important to determine how these hematological changes, which occur in diseased animals, are altered by treatment. Histopathological examination of tissues from treated and untreated virus-infected animals would also be informative in determining which pathological alterations are affected by treatment.

Friend (*25*) previously demonstrated that mice immunized with formalin-inactivated Friend virus were afforded protection against challenge with viable Friend virus and tumor grafts. Fink (*22*, *24*) also reported protection of mice with vaccine prepared with Rauscher virus. In addition, Fink (*22*) reported that homologous immune serum administered 19 to 20 hours before injection of live virus protected against infection.

The results obtained in the present studies indicate that homologous immune serum administered to viremic animals is also capable of reducing viremia and retarding splenomegaly. In two separate experiments immune serum treatment was as effective as 6-MP treatment in reducing virus titer. It is interesting that two treatments with 0.5 ml of immune serum spaced 3 days apart (text-fig. 5) were as effective as 4 treatments with 0.25 ml of immune serum (text-fig. 6) in reducing virus titer. The difference in virus-neutralizing capacity of these two immune serum preparations may be attributable to a difference in potency. In applying immunotherapy as a treatment regimen, it would be important to define the interval at which immune serum should be administered; for instance, treatment every 3d day may be more beneficial than daily treatment.

The results from this study indicate it may be possible to control Rauscher virus infection by combined treatment with drug and homologous immune serum. Certain advantages may be gained by such a regimen of therapy; for instance, when drug treatment must be limited due to the development of host toxicity, therapy may be continued with homologous immune serum. Studies are in progress to determine whether virus replication can be completely suppressed by combined drug and immunotherapy.

SUMMARY

An *in vivo* model system has been developed with the Rauscher virus (RV) to test potential antiviral agents. Antiviral activity is evaluated on the splenomegalic response observed in recipient animals subinoculated with cell-free plasma from virus-inoculated control and treated donor animals.

With this system, the antiviral activity of 6-mercaptopurine (6-MP) and Rauscher virus immune serum (RVIS), alone and in combination, was

evaluated. 6-MP therapy reduced splenomegaly and increased survival time. An early single course of 6-MP treatment resulted in an initial 3.5 log decrease in virus titer. Without subsequent therapy, virus replication progressed in such a manner that 2 weeks after cessation of therapy the virus titer approached that of untreated control values. Two courses of 6-MP therapy, administered at different intervals during the viremic phase, were more effective, resulting in a more sustained decrease in recoverable virus as compared with untreated controls. Treatment of viremic animals with RVIS alone resulted in a reduction of 2.7 logs of virus. Treatment with normal serum was without effect. Treatment with RVIS followed by 6-MP resulted in a greater reduction in virus titer than that achieved with 6-MP therapy alone.

REFERENCES

(*1*) Gross, L.: "Spontaneous" leukemia developing in C_3H mice following inoculation, in infancy, with AK-leukemic extracts, or AK-embryos. Proc Soc Exp Biol Med 76: 27–32, 1951.

(*2*) ———: Oncogenic Viruses. New York, Pergamon Press, 1961.

(*3*) Sinkovics, J. G.: Viral leukemias in mice. Ann Rev Microbiol 16: 75–100, 1962.

(*4*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*5*) ———: The rodent leukemias. Virus-induced murine leukemias. Ann Rev Med 15: 383–392, 1964.

(*6*) Bittner, J. J.: Influence of the mammary-tumor agent on the genesis of mammary cancer in agent-free mice after male transmission. J Nat Cancer Inst 25: 177–199, 1960.

(*7*) Trentin, J. J., Yabe, Y., and Taylor, G.: The quest for human cancer viruses. A new approach to an old problem reveals cancer induction in hamsters by human adenovirus. Science 137: 835–841, 1962.

(*8*) Huebner, R. J., Rowe, W. P., and Lane, W. T.: Oncogenic effects in hamsters of human adenovirus types 12 and 18. Proc Nat Acad Sci USA 48: 2051–2058, 1962.

(*9*) Epstein, M. A., Henle, G., Achong, B. G., and Barr, Y. M.: Morphological and biological studies on a virus in cultured lymphoblasts from Burkitt's lymphoma. J Exp Med 121: 761–770, 1965.

(*10*) Graffi, A., Bierwolf, D., Baumbach, L., Blankenhagel, H., Widmaier, R., and Randt, A.: Virus particles in human myeloid leukemia cells. Deutsche Gesundh 19: 34: 1576–1580, 1964.

(*11*) Bauer, D. J., St. Vincent, L., Kempe, C. H., and Dawnee, A. W.: Prophylactic treatment of smallpox contacts with N-methylisatin β-thiosemicarbazone. Lancet 2: 494–496, 1963.

(*12*) Kaufman, H. E., Nesburn, A. B., and Maloney, E. D.: IDU therapy of herpes simplex. Arch Opthal 67: 585–591, 1962.

(*13*) Stanley, E. D., Muldoon, R. E., Akers, L. W., and Jackson, G. G.: Evaluation of antiviral drugs: The effect of adamantanamine on influenza in volunteers. Ann NY Acad Sci 130: 44–51, 1965.

(*14*) Sugiura, K.: Effects of compounds on the Friend mouse virus leukemia. Gann 50: 251–264, 1959.

(*15*) Mirand, E. A., Back, N., Prentice, T. C. Ambrus, J. L., and Grace, J. T., Jr.: Effect of chemotherapeutic agents on Friend virus-induced leukemia in mice. Proc Soc Exp Biol Med 108: 360–363, 1961.

(*16*) Chirigos, M. A., Luber, E., March, R., and Pettigrew, H.: Antiviral chemotherapeutic assay with Friend leukemia virus in mice. Cancer Chemother Rep 45: 29–33, 1965.

(*17*) Chirigos, M. A., Rauscher, F. J., Kamel, I. A., Fanning, G. R., and Goldin, A.: Studies with the murine leukemogenic Rauscher virus. I. Chemotherapy studies with *in vivo* and *in vitro* assay systems. Cancer Res 23: 762–769, 1963.

(*18*) ———: Studies with the murine leukemogenic Rauscher virus. II. Chemotherapy of virus-induced lymphoid leukemia. Cancer Res 23: 1646–1650, 1963.

(*19*) Chirigos, M. A.: Studies with the murine leukemogenic Rauscher virus. III. An *in vivo* assay for anti-viral agents. Cancer Res 24: 1035–1041, 1964.

(*20*) Chirigos, M. A., Moloney, J. B., Humphreys, S. R., Mantel, N., and Goldin, A.: Response of a virus-induced murine lymphoid leukemia to drug therapy. Cancer Res 21: 803–811, 1961.

(*21*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*22*) Fink, M. A., and Rauscher, F. J.: Immune reactions to a murine leukemia virus. I. Induction of immunity to infection with virus in the natural host. J Nat Cancer Inst 32: 1075–1082, 1964.

(*23*) Rauscher, F. J., and Allen, B. V.: Growth curve of a murine leukemia virus in mice. J Nat Cancer Inst 32: 269–275, 1964.

(*24*) Fink, M. A., Rauscher, F. J., and Chirigos, M. A.: Some immune reactions of murine leukemia virus demonstrable within a completely isologous system. To be published by the National Advisory Cancer Council—3d Workshop Publication on Viral-Induced Tumors—Prospects for Control by Immunological Methods and Chemotherapy. In press.

(*25*) Friend, C.: Immunological relationship of a filterable agent causing a leukemia in adult mice. I. The neutralization of infectivity by specific antiserum. J Exp Med 109: 217–228, 1959.

DISCUSSION

Dr. Barski: I didn't quite understand what the source of your specific anti-Rauscher serum was.

Dr. Chirigos: These are the homologous immune serums we obtained from Dr. Fink that she prepares in her BALB/c mice.

Dr. Barski: Is it a mouse serum?

Dr. Chirigos: Yes.

Chairman Rauscher: What happened when you used other serum?

Dr. Chirigos: We didn't try any other.

Chairman Rauscher: Was primate serum used?

Dr. Chirigos: Dr. Fink reported this in an earlier paper. Would you like to comment on this, Dr. Fink?

Dr. Fink: All of the rodent antisera and the primate antiserum were effective in reducing the viremia; however, the primate serum did not have the capacity for passive immunization when administered 20 hours before the virus.

Dr. Barski: I have another question. Do you have any idea about the long-lasting effect of the serum—I mean when you are introducing your antiserum in mice do you know how long the antibodies are maintained before being eliminated entirely?

Dr. Chirigos: I will have to answer this question by explaining what we think is happening with the drug treatment—anti-immune serum treatment. We feel the antiviral effect we obtained is a cell-directed effect, that is, the drug is apparently decreasing the number of target cells. By target cells I mean the cells that are replicating virus. When you have an effective therapy, you can show by bioassay techniques—(I know you can do this also in the spleen colony assay)—a decrease in virus titer.

This decrease in virus titer reflects a reduction of the cells that are replicating virus. In answer to your question, the mechanism by which the immune serum has exerted its antiviral effect may be in two ways. One definitely acceptable way is the fact that your immune serum is neutralizing free circulating virus. The other possibility is that the immune serum is again directed toward the target cells, those cells that are replicating virus.

This type of hypothesis will have to be substantiated, of course, with further quantitative studies, which we are planning to do.

Dr. Latarjet: Maybe this is relevant to the present paper. To my knowledge, the most powerful way of acting against the development of the Friend disease so far is the injection of radioactive chromium phosphate. This is a colloid, which goes to the reticuloendothelial system. This was done in France 2 years ago by Chevalier and Duplan [C.R. Acad. Sci. (Paris), 256: 4320, 1963].

This is a very puzzling situation; we do not understand how it works. If the radioactive chromium is injected within 5 days after the injection of the virus there is a very strong inhibition of the development of the disease as judged by splenomegaly, hepatomegaly, and survival. However, the viremia is unreduced as if multiplication of the virus was not changed.

If the substance is injected 5 or more days after the infection, there is no effect, which means the effect is not due to the destruction of the malignant cells.

Therefore, we wonder—and we should do some experiments on this point—if, first, this is not an evidence that the cells in which this virus multiplies are different from the cells which will be transformed, and, second, that this local irradiation acts preferentially on those cells competent for malignant transformation.

Protective Effect of Specific Immunization in Rauscher Leukemia [1]

G. Barski [2] and J. K. Youn, *Institut Gustave Roussy, Villejuif (Seine), France, and Sloan-Kettering Institute for Cancer Research, New York, New York*

For nearly 2 years, in our laboratory at the Gustave-Roussy Institut in Villejuif and more recently at the Sloan-Kettering Institute, we have conducted studies aimed at modifying the course of the Rauscher disease by submitting the mice prior to inoculation of the Rauscher virus to two different kinds of treatment:

1. The mice were injected with another virus, the lymphocytic choriomeningitis (LCM) virus in particular; this resulted in what appeared to be an interference phenomenon.

2. They were preinoculated with tissue culture material chronically infected with the Rauscher virus and having an attenuated leukemia-producing capacity; in this case one can assume that the protective effect was due essentially to an immunological phenomenon.

The results of experiments dealing with LCM-Rauscher leukemia interference, briefly reported in a note (*1*), will be described in detail in a separate communication.

Concerning the second point, in a preliminary note (*2*) published recently, we reported that BALB/c mice could be protected to a significant degree against challenge with the Rauscher virus when preinoculated with material from long-term cultures of isologous spleen-thymus cells chronically infected with Rauscher virus. The leukemia-producing capacity of these cultures was rather low and in some instances a complete course of immunization followed by a challenge injection of a fully virulent virus preparation achieved total and long-lasting resistance of the animals to Rauscher leukemia.

The purpose of the investigations reported here was to confirm the previous results and gain more information about the most appropriate material from the infected cultures and the most efficient immunization procedure to use. Furthermore, we wanted to explore the possibility of homologous immunization of mice of strains other than the BALB/c.

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Recipient of the Alfred P. Sloan Foundation Award in Cancer Research, 1965.

MATERIAL AND METHODS

The original strain of Rauscher virus was obtained from Dr. Rauscher. The virus stock currently in use was prepared from a centrifuged homogenate (10% in 0.153 M potassium citrate) of spleen from diseased animals. Its titer, as determined by intraperitoneal inoculations of 3- to 4-week-old BALB/c mice, was of the order of 10^6 LID50 per ml.

One- to 2-month-old BALB/c mice, randombred Swiss mice, or (C58 × BALB/c)F_1 hybrid mice were used. The progress of the Rauscher disease in animals inoculated with the virus intraperitoneally was routinely checked by spleen palpation and also by hematological examinations or determination of spleen weight.

The V5 cell line, developed by Wright and Lasfargues (*3*) from mixed spleen and thymus cultures originating from BALB/c mice, was given to us by Dr. Wright in September 1964. The appearance of the reticulum type cells in these cultures is shown in figures 1 and 2. These cells, chronically infected with the Rauscher virus, constantly produced and released into the culture medium many C-type virus particles and some rare A-type particles. Budding of these particles from the cell surface and evidence of their secondary intake into phagocytic vacuoles were seen frequently (figs. 3 and 4).[3]

The V5 cells were cultivated in our laboratory in a mixture of synthetic medium 109 supplemented with 20% calf serum. The original V5 line cells or cells recovered from the frozen state after preservation for several months at liquid nitrogen temperature (the V5′ line) were used in their eighth to nineteenth passages in our laboratory.

RESULTS

In November 1964, when the supernatant of the V5 cultures was inoculated intraperitoneally at a dose of 0.1 ml per inoculum into 1- to 2-month-old BALB/c mice, no leukemias were observed. Similar negative results were obtained when 1 million cells from the same cultures were inoculated in the same way.

Some leukemias were produced with this material after multiple and more massive inoculations. Thus, after 4 injections totaling 3 ml of the culture supernatant from the V5 cultures (fifteenth passage in our laboratory), from a total of 16 BALB/c mice in two separate experiments, 7 developed leukemia during the 3-month observation period. It is remarkable that when frozen and thawed cells were present in the inoculum (2.5×10^6 cells per inoculum) the incidence of leukemia was significantly lower: 2 in 16 inoculated mice.

---

[3] We wish to thank Dr. Etienne de Harven of the Sloan-Kettering Institute for Cancer Research for taking the electron microscope photographs.

This observation led us to consider the cells from the V5 cultures as an essential source of immunizing material, whether this material was of viral or cellular nature.

The results of one of our first experiments in checking the immunizing potential of the V5 culture supernatant as compared with the cells from the same cultures are listed in table 1. The immunization procedure was as follows: Mice were given combined intraperitoneal (0.5 ml) and intramuscular (0.25 ml) injections of either centrifuged culture supernatant or similar injections of the supernatant containing in the same volume approximately 2.5×10^6 cells, frozen and thawed 3 times. The injections were given on the 1st, 4th, and 7th days and were followed by a booster intraperitoneal injection on the 22d day. The mice were challenged 7 days later.

As shown in table 1, the assumption that mice previously treated with material from V5 cultures would be immune against challenge with virulent Rauscher virus was entirely confirmed. Furthermore, cells from these cultures appeared to be more efficient as immunizing material than the culture supernatant.

These results have remained practically unchanged since they were first reported 55 days after challenge (*2*). All the 19 mice immunized with the cells appeared to be definitely protected.

To check the efficiency and permanency of the state of induced immunity, 92 days after the first challenge inoculation, surviving mice in all the experimental groups were challenged again with an intraperitoneal injection of a double dose of the active stock virus (table 2).

One can conclude from these data that mice immunized with the tissue culture material that had resisted the first challenge maintained their resistance to further inoculations of the virulent virus. This immunity lasts for 3 months and probably covers the lifespan of the animals, since it blends with the natural resistance to the Rauscher virus which increases with age.

TABLE 1.—Immunization of BALB/c mice against Rauscher leukemia with V5 cell culture material

Immunizing material from V5 cultures	Challenge with virulent Rauscher virus (10^5LD50)	Proportion of animals (with splenomegaly) observed after:	
		55 days	90 days
Supernatant	−	3/9	3/9 (33%)
Supernatant plus cells	−	0/10	2/10 (20%)
Plain medium (controls)	+	8/10	8/10 (80%)
Supernatant	+	9/19	9/19 (47%)
Supernatant plus cells	+	0/19	0/19 (0%)

TABLE 2.—Resistance to second challenge in BALB/c mice previously immunized with tissue culture material

Previous treatment with 3 immunizing plus 1 booster injection of:	First challenge with Rauscher virus	Incidence of leukemia 92 days later	Number of mice surviving and challenged for the second time	Incidence of leukemia 60 days after the second challenge
Culture supernatant	+	9/19	10	0/10
Frozen cells	+	0/19	19	0/19
Culture supernatant	—	3/9	6	1/6
Frozen cells	—	2/10	8	0/8
Plain culture medium	+	8/10	2	1/2

We next checked to see whether the V5 cell cultures, originally developed from BALB/c tissues, could be used to induce resistance to Rauscher virus not only in BALB/c mice but also in homologous mice.

In two series of experiments, 1- to 2-month-old Swiss mice were injected either with living or frozen cells from V5 cultures or the supernatant from these cultures. The results of challenge of these mice with virulent Rauscher virus are reported in table 3.

It is evident from the results of these two experimental series that efficient protection can be obtained in Swiss mice by preventive inoculation of either living or frozen cells. Furthermore, it appears that a single subcutaneous injection of 3 million living cells can produce full protection against a challenge with virulent Rauscher virus 7 or 29 days later. Here

TABLE 3.—Immunization of Swiss mice against Rauscher virus leukemia with homologous Rauscher-virus-infected tissue culture material

Experiment No.	Material from V5 cultures used for immunization	Number of injections (subcutaneous)	Challenge with virulent Rauscher virus after: (days)	Observed at: (days)	Incidence of leukemia
Villejuif 5	Living cells (3×10^6)	1	—	78	0/7
	Living cells (3×10^6)	1	7	70	0/7
	Living cells (3×10^6)	1	29	70	0/7
	Plain tissue culture medium (control)	1	7 or 29	56	9/14
Sloan-Kettering Institute 1	Culture supernatant (0.5 ml)	3 (every 4th day)	17	104	1/8
	3× frozen and thawed cells (3×10^6)	3	17	104	0/8
	Living cells	1	17	104	0/8
	No treatment (controls)	—	+	104	10/10*

*Nine of the 10 mice with splenomegaly died before 3 months; their spleen weights ranged from 1.4 to 2.5 g (averaging 1.85 g).

again, the culture supernatant appears as a less efficient immunizing material than the V5 cells.

Some experiments were undertaken with V5′ cultures recovered from the frozen state after several months' preservation at liquid nitrogen temperature. These cultures, when used for immunization in their tenth to twelfth *in vitro* passages after freezing, differed to some extent from the original V5 line in having a more pronounced leukemia-producing and a lesser immunizing capacity.

These cultures were used to compare the protective effect of inoculation with frozen and thawed, living, or irradiated (9000 R) cells. Following the procedure previously used, we gave BALB/c males, 45 to 60 days old, 3 intraperitoneal and intramuscular immunizing injections followed by 1 intraperitoneal booster injection and challenged them 8 days later with the virulent virus.

From the results presented in table 4, it can be concluded that despite the fact that the V5′ cultures had shown a considerable leukemia-producing capacity by themselves, the previous observations concerning the protective effect conferred on the animals by preinoculation with these cells were largely confirmed.

In this experiment, inoculation of irradiated cells appeared to be the most efficient method of immunization. It is remarkable that the proportion of animals developing leukemia is of the same order in groups #3 and 4, both of which were inoculated with the irradiated cells and challenged or unchallenged with the virulent virus. We repeatedly observed this apparently paradoxical phenomenon: The proportion of diseased animals in the immunized and challenged group was the same or even lower than in the control group that was immunized and not challenged. It was as if the challenge injection of the virulent virus, coming after the course of immunization, actually boosted the immunological reaction of the animal. Similar reactions were reported in children immunized with polio vaccine and later exposed to natural superinfection with the virulent virus.

Confirmation of the data on the value of irradiated cells as immunizing material was obtained in another series of experiments performed at the Sloan-Kettering Institute in cooperation with Dr. Burchenal and M.

TABLE 4.—Comparative results of immunization of BALB/c mice with V5′ cells: frozen, living, or irradiated

Group	Immunizing material	Challenge with virulent Rauscher virus	Proportion of animals with pronounced splenomegaly after: 31 days	42 days	59 days
1	Frozen cells	+	5/13	4/13*	4/13 (31%)
2	Living cells	+	6/18	7/18	8/18 (44%)
3	Irradiated cells	+	5/23	5/23	5/23 (22%)
4	Irradiated cells	−	2/17	2/17	3/17 (18%)
5	Plain, medium (controls)	+	16/17	16/17	17/17 (100%)

*In 1 mouse the splenomegaly regressed.

Haidri[4] using (C58 × BALB/c)F_1 mice. These mice were highly receptive to Rauscher virus, developing splenomegaly, starting from the 10th day after inoculation, and Rauscher leukemia at least as rapidly as the parental BALB/c mice. Table 5 summarizes the results obtained when control, untreated, 6- to 7-week-old (C58 × BALB/c)F_1 mice and similar mice inoculated previously once or 3 times with V5 cells were challenged 10 days after the last immunizing injection with the virulent Rauscher virus. Three million V5 cells either living or irradiated (5000 R) were used for the subcutaneous immunizing injections.

TABLE 5.—Comparative results of immunization of (C58 × BALB/c)F_1 mice with frozen or irradiated V5 cells or with the culture supernatant (preliminary data)

Group	Immunizing material	Number of immunizing injections	Challenge with virulent Rauscher virus	Proportion of animals with splenomegaly	
				Day after challenge	
2B	Living cells	4	+	43	0/10
3B	Living cells	1	+	65	0/8
6B	Irradiated cells	4	+	24	0/10
7B	Irradiated cells	1	+	50	0/10
4B	Culture supernatant	4	+	43	0/7
5B	Culture supernatant	1	+	65	2/7
8–9	Controls (untreated)	—	+	43	20/20

Though it is too early to draw definite conclusions from these experiments, it appears that irradiated cells were clearly at least as efficient as living cells in preventive immunization of the (C58 × BALB/c)F_1 mice. Once more, the cells from the cultures were superior to the culture supernatant in inducing immunity.

DISCUSSION AND CONCLUSIONS

The V5 long-term, mixed spleen and thymus cell cultures chronically infected with Rauscher virus (*3*) represent an example of a well-equilibrated *in vitro* carrier system in which a virus multiplies permanently and is constantly released by a proliferating cell culture.

Similar systems showing no evidence of cytolytic effect involving cell cultures latently infected with nononcogenic viruses, such as the Newcastle disease virus (*4*), myxoviruses (*5*), coxsackie virus (*6*), polio virus (*7*), or arborviruses (*8*), were reported by many workers. We recently described a carrier system comprising mouse cell cultures and an oncogenic agent, the polyoma virus (*9*).

However, our new finding, confirming the results of Dr. Wright (*see* paper, this Monograph), is that the viral material produced in the V5 cultures somehow has a decreased leukemogenic potential.

---

[4] Experiments now in progress.

The nature of this decrease is so far not well understood. It could be due to a partial or total substitution of the "normal" Rauscher virus by a less leukemogenic viral mutant. This would be a situation similar to that existing when, for example, a neurotropic polio virus is substituted *in vitro* or *in vivo* by a non-neurotropic variant of the same virus.

Another possible explanation of the "attenuation" phenomenon is that cells in V5 cultures are producing huge quantities of an "incomplete," noninfectious but fully antigenic virus along with some complete and infectious virus particles. Both immunological and interference phenomena can be responsible then for the "attenuation" of the cultures.

The initial assumption that preinoculation with material from the "attenuated" V5 cultures can have a protective effect against further challenge with the virulent Rauscher virus was fully confirmed by data obtained during the last year, by parallel studies in our Villejuif Laboratory and at Sloan-Kettering Institute.

Good evidence of this protective effect was obtained not only in BALB/c mice but also in (C58 × BALB/c)F_1 and homologous Swiss mice.

The protective effect was long lasting as evidenced in animals challenged 29 days after treatment with the V5 cells in the Villejuif experiment #5 and also when the protected animals were challenged again with the virulent Rauscher virus (experiment listed in table 1). This suggests an immunological protection phenomenon rather than one of an interference-like nature. As a matter of fact, this situation is in contrast to that existing when the protective effect was obtained in previous experiments by inoculation of BALB/c mice with the interfering lymphocytic choriomeningitis (LCM) virus prior to challenge with Rauscher virus (*1*). When the LCM-pretreated mice, not developing leukemia for 3 months after challenge with Rauscher virus, were challenged again, they all developed typical Rauscher leukemia (*10*).

On the whole, the supernatant as well as the cells from V5 cultures, both containing large amounts of C-type virus particles, have shown a residual leukemia-producing capacity. However, apparently, in several experiments, the additional presence of V5 cells in the inoculated material usually did not increase the incidence of leukemia but, on the contrary, frequently decreased it.

Accordingly, in the immunization assays the cells from the V5 cultures, whether frozen or irradiated, generally induced immunity in the inoculated animals more efficiently than the culture supernatant.

Further experimentation is needed to decide whether this is due to the fact that the cells simply represent a more abundant source of the immunizing factor in the form of attenuated or incomplete virus. An alternative explanation would be that the spleen-thymus cells infected *in vitro* with the Rauscher virus contain a new antigen also present in the virus-transformed cells *in vivo*, which is essential in the immunological protection of the animal against the progress of the Rauscher disease. Experiments now in progress have been designed to test the validity of this concept.

SUMMARY

Long-term, *in vitro* mixed spleen and thymus cell cultures from BALB/c mice chronically infected with Rauscher leukemia virus had a decreased leukemogenic activity. C-type and a few A-type particles continuously produced in these cultures could be seen budding from the cell surface and accumulating in the culture medium as well as inside cell vacuoles. The medium or cells from these cultures when inoculated into BALB/c mice had very attenuated leukemia-producing capacity. Furthermore, mice treated with this material resisted challenge with virulent Rauscher virus. A more detailed study demonstrated that BALB/c and also homologous or hybrid mice could be successfully immunized against Rauscher leukemia by using these cultures and different immunization procedures. Cells from the cultures appeared to be a more efficient immunizing material than the virus-containing culture medium. Frozen, living, or irradiated cells could be used for the immunization. The acquired immunity was long lasting, extending over a period of at least 3 months.

REFERENCES

(*1*) Barski, G., and Youn, J. K.: Interférence entre la chorioméningite lymphocytaire et la leucémie de souris de Rauscher. C R Acad Sci (Paris) 259: 4191–4194, 1964.

(*2*) ———: Immunization against Rauscher mouse leukemia with tissue culture material. Science 149: 751–752, 1965.

(*3*) Wright, B. S., and Lasfargues, T. C.: Multiplication of the Rauscher leukemia virus in a mixed culture of mouse spleen and thymus cells. Proc Amer Assoc Cancer Res 5: 70, 1964.

(*4*) Mason, E. J., and Kaufman, N.: The persistent production of small quantities of infectious Newcastle disease virus in grossly unaltered L and U_{12} strain cells. J Immun 86: 413–420, 1961.

(*5*) Henle, W., Henle, G., Deinhardt, F., and Bergs, V. V.: Studies on persistent infections of tissue culture. IV. Evidence for the production of an interferon in MCN cells by myxoviruses. J Exp Med 110: 525–541, 1959.

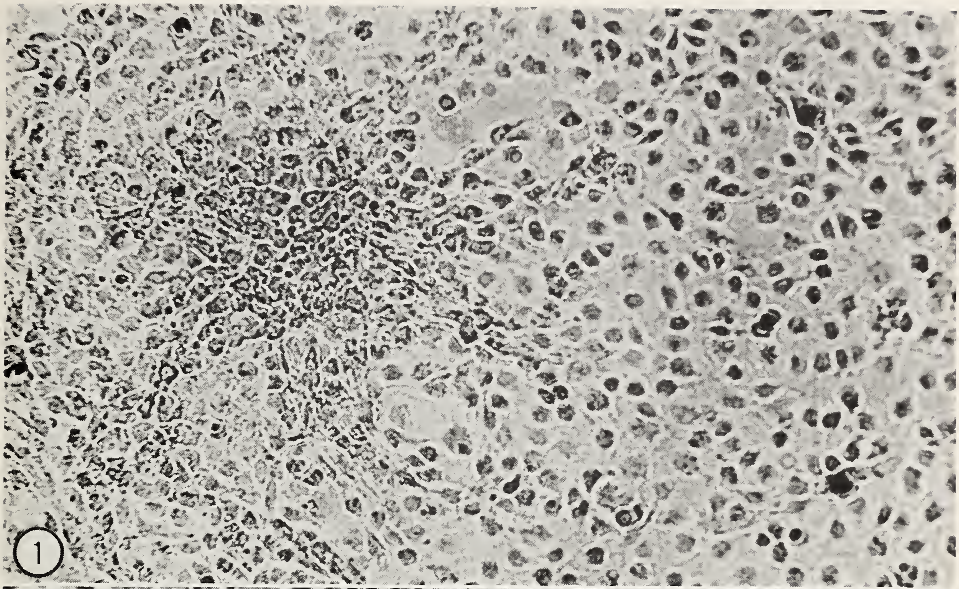
(*6*) Takemoto, K. K., and Habel, K.: Virus-cell relationship in a carrier culture of HeLa cells and Coxsackie Ag virus. Virology 7: 28–44, 1959.

(*7*) Ho, M., and Enders, J. F.: Further studies on an inhibitor of viral activity appearing in infected cell cultures and its role in chronic viral infections. Virology 9: 446–477, 1959.

(*8*) Lockart, R. Z. J.: The production and maintenance of virus carrier state: the possible role of homologous interference. Virology 10: 198–210, 1960.

(*9*) Barski, G., and Cornefert, F.: Response of different mouse cell strains of polyoma infection *in vitro*. Latency and self-inhibition effect in infected cultures. J Nat Cancer Inst 28: 823–843, 1962.

(*10*) Youn, J. K., and Barski, G.: Unpublished data.



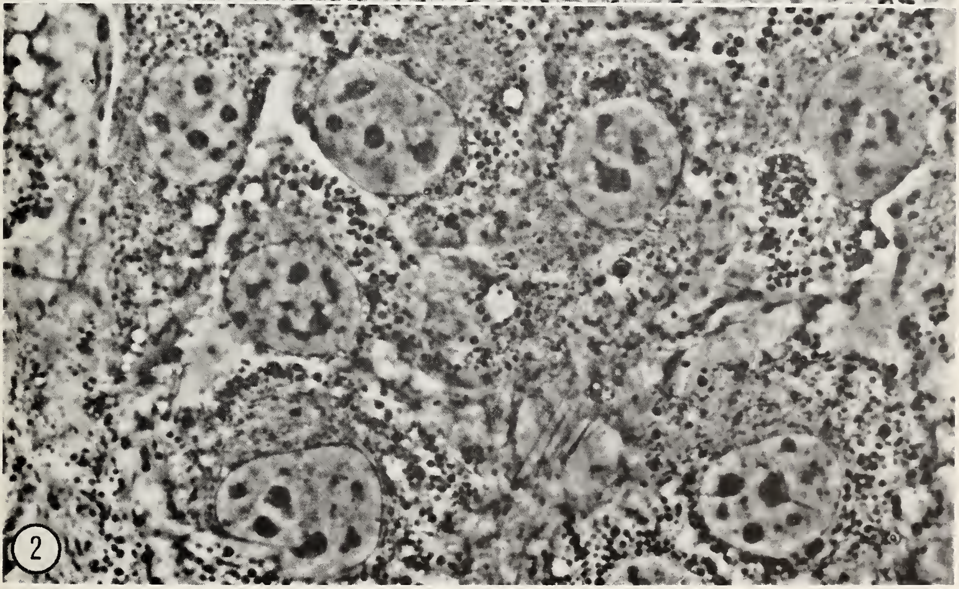
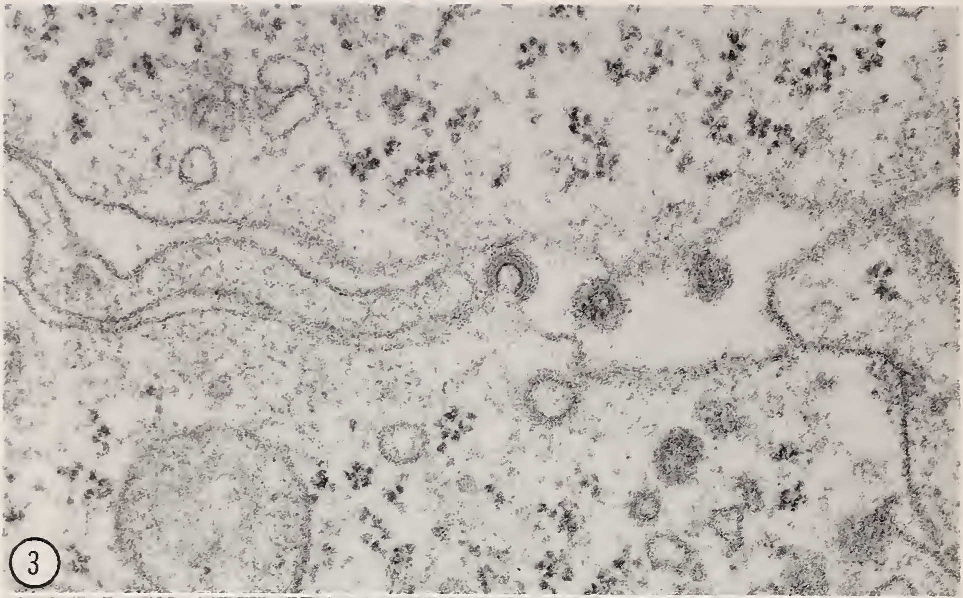


FIGURE 1.—Cells of the V5 spleen-thymus culture infected with Rauscher virus. × 10

FIGURE 2.—Cells of the V5 spleen-thymus culture infected with Rauscher virus. Phase contrast. × 100



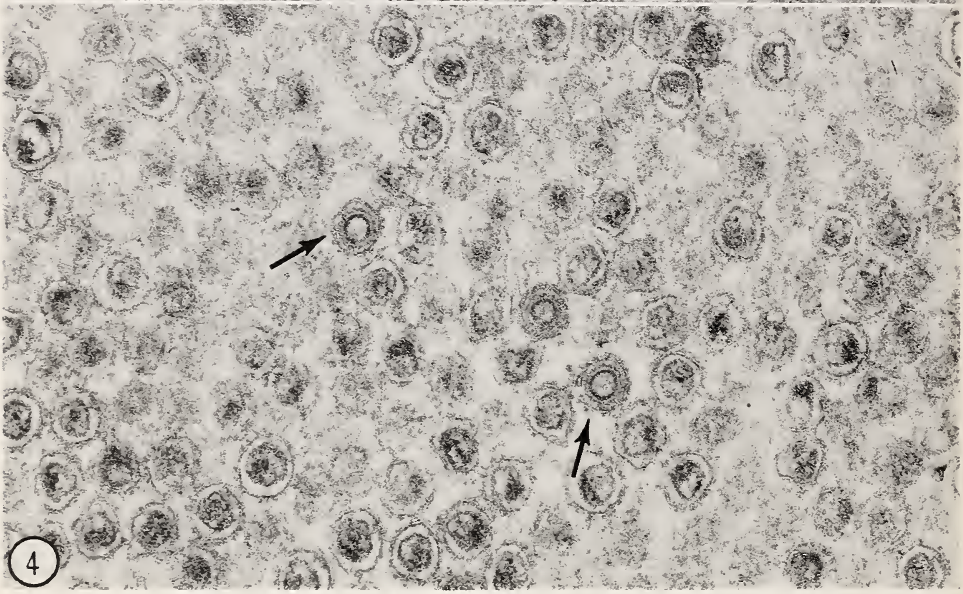


FIGURE 3.—Tissue culture of V5 cell line showing a typical budding particle at level of plasma membrane of an infected cell. × 77,000

FIGURE 4.—Thin section of pellet prepared from supernatant of a V5 tissue culture. A large collection of virus particles is observed, most of them being of the C type and a few of the A-1 type (*arrows*). × 88,000

DISCUSSION

Dr. Wright: In your studies did you use any of the V6 cells as a protective agent?

Dr. Barski: No, we did not.

Dr. Sykes: From passages 176, 190, and 232 of the original V5 culture which we got from Dr. Wright—this is the actual passage number—we have prepared high-speed pellets from sonic-vibrated cells. We suspended the cell-free, high-speed pellets to the same volume as the tissue culture fluids from which they originated and diluted this material 10^{-2}. With it we obtained a very high proportion of Rauscher leukemia within 28 to 58 days in our inoculated C3Hf/EB/FeJ mice.

In an earlier passage (I think it was around 150 or so), we used 10^5 washed cells per mouse and got a 100% incidence of Rauscher leukemia. The results we obtained may possibly be due to our tissue culture medium, or to the strain of mice used for bioassay.

Dr. Barski: What was the age of your mice?

Dr. Sykes: Mice were inoculated at 4 to 9 days of age. I might add for Dr. Wright's benefit that similar studies with V6 have been completely negative.

Dr. Fink: We have published the results of a considerable amount of similar experimentation showing that the BALB/c mouse can be immunized very solidly to the Rauscher disease by the injection of formalin-treated virus. We also have some unpublished results that I believe are pertinent. On several occasions, we immunized infected, transplantable ascites cells with the Rauscher virus and found that the amount of immunity produced was a reflection of the amount of virus present in the cells and is a function of the amount of neutralizing antiviral antibody in the circulation.

Therefore, I would like to ask Dr. Barski two questions. When cells were included in the immunizing inoculum, was the amount of virus in these cells assessed, and was it in excess of what he used when he used only the supernatant? Secondly, has he made any attempt to determine the amount of circulating antibody to the virus in the immunized mice?

Dr. Barski: Concerning the first question, actually the difference between immunization with the supernatant and immunization with cells was that the cells were additionally present in the supernatant; in the latter the protection was better. However, the incidence of leukemias produced with the supernatant by itself was higher than with cells and we are postulating the presence in the cells of an immunizing antigen other than infectious virus.

Concerning the presence of antibodies, we know there are antibodies in the protected mice, but we did not check their exact titer.

Dr. Chieco-Bianchi: The age of the animals at immunization is a very important factor. By using Graffi virus we obtained data indicating that a state of immunological tolerance to the virus can be induced only in newborn or very young mice. Obviously, it is quite difficult to produce an effective immunization in these tolerant animals. These results are in agreement with those reported by Dr. Axelrad in the AKR system (Progr. Exp. Tumor Res., vol. 6, pp. 30–83, Karger, Basel and New York, 1965) and by Dr. Klein with Moloney virus at this Conference.

Studies of Moloney and Rauscher Leukemia Viruses in Cell Cultures by Immunofluorescence [1, 2]

DONALD PINKEL,[3] KIYOZO YOSHIDA, and KIRBY SMITH, *Laboratory of Virology, St. Jude Children's Research Hospital, Department of Pediatrics, University of Tennessee, Memphis, Tennessee*

AN *in vitro* assay method for murine leukemia viruses has been needed, both for investigation into the nature and distribution of these viruses and for use in the search for possible human leukemia viruses. The failure of murine leukemia viruses to produce cytopathic or transforming effects *in vitro* has hampered the development of such a method (*1*).

Recently, we have adapted the indirect immunofluorescence method to the detection of Moloney leukemia virus (MLV) and Rauscher leukemia virus (RLV) in cells from infected animals and in infected cell cultures (*2*). Rabbit antiviral sera and fluorescein isothiocyanate conjugated goat antirabbit globulin globulin (FITC-GRGG) produced specific fluorescence limited to the cytoplasm, and there was no evidence of cross-antigenicity between the two viruses. Immunofluorescence also was useful for demonstration of RLV and MLV replication in cell cultures. On the basis of these observations, efforts were initiated to develop an *in vitro* assay method for MLV and RLV. The methods and preliminary results are described and discussed in this report.

MATERIALS AND METHODS

Virus preparations.—Plasma from RLV- or MLV-infected BALB/c mice or plasma concentrates of these viruses were diluted in Dulbecco's phosphate-buffered saline (PBS), *p*H 7.3, and used to infect cell mono-

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] This investigation was supported by Public Health Service research grants CA-07054 and 1-Tl-CA-5176 from the National Cancer Institute, and by American Lebanese Syrian Associated Charities (ALSAC).

[3] Appreciation is expressed to Dr. Allan Granoff for his advice and to Mr. Aubrey Howard, Mr. Stanley Dabrowski, and Mr. Maurice Beckwith for their technical assistance.

layers. The plasma concentrates were obtained through the courtesy of Dr. J. Moloney, Dr. F. Rauscher, and Dr. S. Mayyasi and were prepared in The John L. Smith Laboratory, Maywood, New Jersey, according to the method of Moloney (*3*). The virus preparations were stored at −70° C until just before use.

Virus inoculation of cell cultures and determination of percent immunofluorescent cells.—Cell monolayers consisted of secondary cultures of mouse fetal cells (MFC) derived from 3-day-old primary cell cultures of trypsinized 12- to 17-day-old BALB/c mouse fetuses. The growth medium was made up of minimal Eagle's medium containing 0.05% yeast extract, 0.25% lactalbumin hydrolysate, 0.5% bactopeptone (w/v), and 10% fetal calf serum (v/v). Penicillin, 100 units per ml, streptomycin, 50 μg per ml, and amphotericin, 1.5 μg per ml, were added, and the *p*H was adjusted to 7.4 with 10% $NaHCO_3$ solution. For maintenance the serum concentration was reduced to 2 or 3%.

In early experiments designed to determine the pattern of appearance and progression of immunofluorescence in infected cell monolayers, 4 ml volumes of a cell suspension containing 1×10^5 cells per ml in growth medium were plated in 55 mm diameter plastic petri dishes (Falcon) with two 18 mm diameter glass coverslips in each dish. Three days later, 0.2 ml of a serial tenfold dilution of virus was inoculated onto each test monolayer. After the cultures were incubated for 2 hours at 37° C for attachment of virus, 4 ml of maintenance medium was added to each dish and the cultures were returned to 37° C. Coverslips were removed from 2 cultures at each virus dilution and from 2 uninfected control cultures on the 1st day after inoculation and on alternate days thereafter for 31 days. Three of the 4 coverslips removed from each pair of cultures were tested for immunofluorescence and the percentage of fluorescent cells was determined by counting 200 randomly distributed cells on each coverslip. The remaining coverslip was stained by the Wright-Giemsa method and examined by light microscopy for possible morphological changes associated with virus infection. Residual cells on the 2 dishes were scraped off and centrifuged to form pellets for ultrathin sectioning and electron microscopic examination. Because cultures were kept for up to 31 days, trypsinization and replating of the cells were required every 9 to 11 days to maintain the monolayers in a healthy state.

For the *in vitro* assay studies, Leighton tubes with 10 mm × 55 mm coverslips were used. One ml volumes of a 5×10^4 cells per ml suspension in growth medium were plated in the tubes, and the resultant monolayers maintained *without trypsinization* for the full length of the experiments, up to 15 days. The cultures were infected only 1 day after the cells were plated, before formation of confluent monolayers, so that the monolayers would remain intact without trypsinization for the duration of the tests. After aspiration of medium, 0.1 ml of a serial dilution of virus was inoculated into each tube, and after 2 hours' incubation at 37° C, 0.9 ml of maintenance medium was added and the cultures were returned to 37° C. Three tubes were used for each virus dilution and 3 for the uninfected controls. For the MLV assay, 3 coverslips at each virus dilution were removed at 15 days for immunofluorescence testing, whereas, for the RLV assay, 3 at each virus dilution were removed at 10 days. Six hundred cells were counted at random on each coverslip to determine the percentage of fluorescent cells.

In additional assay experiments designed to test the feasibility of an endpoint dilution method, 5 Leighton-tube-MFC cultures were inoculated with each serial dilution of virus and tested for immunofluorescence after the same incubation times as above. Coverslips with 0.5% or more fluorescent cells were considered positive and the 50% endpoint was calculated by the method of Reed and Muench (*4*).

All cultures in these experiments were incubated at 37° C in a humidified atmosphere of 3% CO_2 in air and their media were changed twice weekly.

Immunofluorescence methods.—The preparation and evaluation of the rabbit antiviral sera and the techniques used for indirect immunofluorescence tests of cell monolayers with the antisera and FITC-GRGG were described in a previous report (*2*).

Preparation of cells and tissues for electron microscopy and estimates of virus-infected cells.—The preparation methods were described in a previous publication (*2*). For each preparation, 50 cells on 3 grids were counted at random and the proportion of cells with virus particles at their surfaces was calculated.

RESULTS

Appearance and Progression of Immunofluorescence in Virus-Inoculated Monolayer

Immunofluorescent cells first appeared 11 days after inoculation in petri-dish MFC monolayers inoculated with MLV plasma concentrate, undiluted or at 10^{-1} dilution (table 1). At higher dilutions of virus inocula, immunofluorescent cells appeared later. At all dilutions the percentage of fluorescent cells tended to increase for 10 days after initial appearance of fluorescence and then to remain constant or diminish. Similar results were seen with RLV-infected MFC monolayers, except that initial immunofluorescence appeared 1 day after inoculation and by 11 days even the monolayers inoculated with the 10^{-6} virus dilution demonstrated fluorescence (table 2). Although some fluorescent cells were grouped together, many were scattered randomly in the monolayers (fig. 1). Fluorescence appeared first in the perinuclear areas of infected cells and later in the peripheral cytoplasm (fig. 2). Among the fluorescent cells were some in mitosis (fig. 3), which indicated that virus and/or virus antigen can be transmitted from parent to daughter cells.

TABLE 1.—Appearance and progression of immunofluorescence in Moloney leukemia virus-infected monolayers*

Virus dilution	% immunofluorescent cells																
	Days after inoculation:																
	0	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
10^0	0	0	0	0	0	0	3(2–4)	12(11–15)	15(14–19)	18(17–19)	26(25–27)	32(28–34)	33(32–34)	39(36–44)	34(32–36)	X	31(28–36)
10^{-1}	0	0	0	0	0	0	1(0.5–1)	9(8–10)	11(10–13)	14(10–17)	23(22–24)	28(26–30)	29(28–32)	25(23–26)	23(20–28)	X	21(19–24)
10^{-2}	0	0	0	0	0	0	0	4(3–5)	6(5–7)	8(7–8)	19(15–22)	23(20–27)	26(24–29)	27(26–28)	29(26–32)	39(36–44)	30(28–33)
10^{-3}	0	0	0	0	0	0	0	1(1–1.5)	2(0.5–3.3)	6(4–7)	12(10–15)	21(18–25)	24(23–26)	27(25–30)	33(32–35)	39(36–42)	33(32–37)
10^{-4}	0	0	0	0	0	0	0	0	0	1(0.5–1.5)	11(7–14)	12(11–13)	14(12–16)	18(15–20)	30(28–32)	34(32–36)	29(28–30)
10^{-5}	0	0	0	0	0	0	0	0	0	0	3(2–4)	8(6–10)	12(10–15)	14(12–16)	X	X	X
PBS control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Each value is average of 3 determinations, each of which is based on count of 200 cells on one coverslip. Range of determinations is in parentheses. X's represent cultures lost because of degenerative changes or contamination.

TABLE 2.—Appearance and progression of immunofluorescence in Rauscher leukemia virus-infected monolayers*

Virus dilution	% immunofluorescent cells														
	Days after inoculation:														
	0	1	3	5	7	9	11	13	15	17	19	21	23	25	27
10^0	0	1(0.5–1)	2(1.5–3)	12(9–12)	26(22–29)	30(30–30.5)	33(31–36.5)	35(31.5–36)	X	X	29(28–30.5)	X	X	17(14–19)	X
10^{-1}	0	0	0	8(5–11)	18(17.5–18)	25(22–26.5)	30(29–30.5)	32(30–36.5)	X	52(45.5–57)	X	45(42.5–47)	X	X	18(17–20)
10^{-2}	X	0	0	1(0.5–2)	10(8.5–10.5)	23(20.5–24.5)	25(22–26.5)	32(29.5–33)	X	42(37.5–50.5)	47(39.5–49.5)	X	29(26–33)	X	18(16–19)
10^{-3}	X	X	X	0	5(4.5–6)	16(14.5–21.5)	19(17.5–20.5)	23(20.5–24)	X	34(30–35)	40(39–40.5)	X	28(25.5–33.5)	X	19(16.5–23)
10^{-4}	X	X	X	0	0	10(8.5–10.5)	12(11–13)	13(11.5–13.5)	X	27(22–29)	32(27.5–30.5)	29(25–34)	X	18(16–20)	16(13–20)
10^{-5}	X	X	X	0	0	4(3.5–4.5)	7(5.5–10)	13(12.5–14.5)	X	26(25–27.5)	32(29–33.5)	24(19–29)	18(15–20.5)	14(12–14.5)	10(9.5–10.5)
10^{-6}	X	X	X	X	0	0	5(3.5–7)	9(8.5–9.5)	X	18(14.5–21)	20(18.5–21)	23(21.5–24)	17(14.5–18.5)	14(13–16.5)	10(8.5–11.5)
Control	0	X	0	X	X	0	X	X	X	X	X	X	X	X	X

*Each value is average of 3 determinations, each of which is based on count of 400 cells on one coverslip. Range of determinations is in parentheses. X's represent cultures deleted since they were unnecessary for the experiment or cultures lost because of degenerative changes or contamination.

Thirteen to 17 days after inoculation the percentage of immunofluorescent cells in MLV-infected cultures bore a straight-line relationship to the $\log_{10}$ concentration of virus inoculum. The same phenomenon was observed with RLV-infected cultures 5 to 11 days after inoculation. This relationship tended to diminish with increasing length of incubation. Repeat experiments with different virus preparations confirmed these patterns of appearance and spread of immunofluorescence, although actual percentages of fluorescent cells differed.

Concurrent electron microscope examination of cell pellets from MLV infected and control cultures demonstrated that virus particles first became apparent in the cell cultures inoculated with the undiluted virus preparation 11 days after inoculation. Despite the sampling problem with ultrathin-sectioned tissues, the proportion of cells with virus particles at their cell membranes generally reflected the percentage of immunofluorescent cells in preparations made from the same culture. Figures 2 and 4 represent preparations made from the same cell culture and illustrate this association between immunofluorescence and virus formation. In no instances were virus particles seen in absence of fluorescence, although in some cultures with few fluorescent cells no virus particles were observed in the sections examined. This can be attributed to the sampling problem. Control cell cultures were consistently negative for virus particles and immunofluorescence.

Susceptible BALB/c mice were given injections of media from infected cultures to determine their leukemogenic potency. Media from cultures inoculated 11 days previously with 10^0 to 10^{-4} dilutions of RLV produced typical disease in BALB/c mice in 60 days. Similar experiments with media from MLV-infected cultures are in progress.

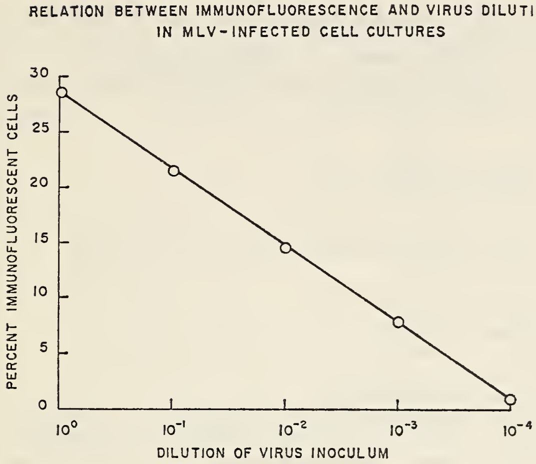
These findings show that the appearance and progression of immunofluorescence in the cell monolayers represented initiation and progression of virus production as well as virus antigen formation in the monolayers.

Morphology of Virus-Infected Cells

The mouse fetal cells used were a mixture of epithelial cells and fibroblasts. Virus-infected and control cells appeared similar both by light microscopy of Wright-Giemsa stained and unstained coverslips and by electron microscopy of ultrathin sections. Both infected and control cell cultures showed degenerative changes with increasing length of incubation.

In Vitro Assay of MLV and RLV by Immunofluorescence

The observation of a relationship between percentage of immunofluorescent cells and dilution of virus inoculum in the above experiments led to efforts to develop an assay method for MLV and RLV based on this observation. Fifteen days after infection of Leighton-tube-MFC monolayers with MLV, the percentages of fluorescent cells in the monolayers

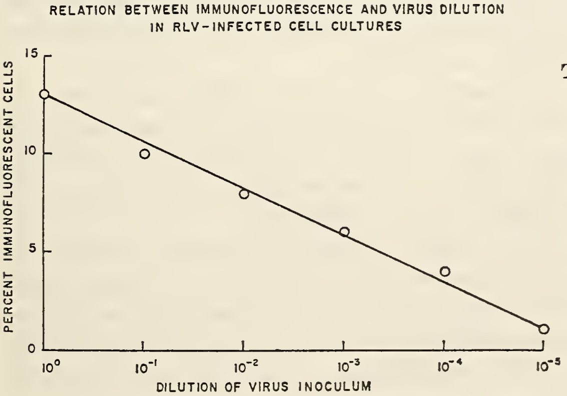


TEXT-FIGURE 1.—MLV-infected MFC monolayers in Leighton tubes were tested for immunofluorescence 15 days after inoculation. Each value is average of 3 determinations, each of which is based on count of 600 cells on one coverslip.

bore a straight-line relationship to the $\log_{10}$ dilution of virus inoculum (text-fig. 1). This was observed in 3 separate experiments. In 2 of the experiments the same virus preparation was used with similar results.

The same straight-line relationship between immunofluorescence and virus dilution was obtained with RLV-infected monolayers stained 10 days after inoculation (text-fig. 2).

Because tenfold differences in virus inoculum were reflected by relatively small differences in percentage of fluorescent cells, it was apparent that the percentage of fluorescent cells could not be used as a measure of infectious virus, as in the method described for Newcastle disease virus assay (*5*). Therefore, further efforts were directed toward development of a 50% endpoint dilution test with immunofluorescence replacing cytopathic effect as the endpoint. Results of preliminary experiments are shown in table 3. The 50% fluorescent cell dose (FCD50) is defined as the unit of virus that will produce 0.5% or more fluorescent cells in one half of the cultures tested 15 days after virus inoculation for MLV and



TEXT-FIGURE 2.—RLV-infected MFC monolayers in Leighton tubes were tested for immunofluorescence 10 days after inoculation. Each value is average of 3 determinations, each of which is based on count of 600 cells on one coverslip.

TABLE 3.—Titration of MLV* and RLV* by immunofluorescence in cell culture†

Virus	Dilution	Number positive / Number inoculated	50% fluorescent cell doses (FCD50) per ml
MLV	10^{-3}	5/5	$10^{5.2}$
	10^{-4}	3/5	
	10^{-5}	0/5	
RLV	10^{-4}	5/5	$10^{5.8}$
	10^{-5}	2/5	
	10^{-6}	0/5	

*MLV = Moloney leukemia virus; RLV = Rauscher leukemia virus.

†Cell monolayers on coverslips were stained for immunofluorescence at 15 days after virus inoculation for MLV and at 10 days for RLV. Cultures with 0.5% or more fluorescent cells were considered positive. Cultures at lower dilutions (not shown) were all positive. Fifty percent endpoints were calculated by the method of Reed and Muench.

10 days after inoculation for RLV. A repeat titration of the MLV sample yielded the same result, but further studies are necessary to evaluate the sensitivity, reproducibility, and practical usefulness of this test.

DISCUSSION

The use of immunofluorescence in cell culture is convenient for the detection of MLV and RLV and will be valuable in the study of murine leukemia and in the search for MLV and RLV or antigenically related viruses in patients with leukemia. If, as preliminary results indicate, it can be adapted to measurement of MLV and RLV by an endpoint dilution method, it will provide a simple, rapid method for quantitative assay of these viruses and their corresponding antibodies.

Previous work (*2*) demonstrated the lack of cross-antigenicity of MLV and RLV in our immunofluorescence method, so that this test distinguishes between the two viruses, in contrast to the complement-fixation and tissue-culture assay method of Hartley *et al.* (*6*) in which CF antigens common to MLV- and RLV-infected cells were found.

It is not known why all the cells in the infected MFC monolayers do not become immunofluorescent and why the percentages of immunofluorescent cells in the monolayers often are less after prolonged incubation. One explanation is that interferon or interferon-like inhibitors, as described by Sinkovics and Howe (*7*), are produced in cultures of MLV- and RLV-infected spleen cells. Other possible explanations are that the cell population of secondary mouse fetal cell cultures is heterogeneous and not all cell types support virus replication, that the repeated trypsinization of the monolayers removes susceptible or infected cells, that senescent monolayers fail to support virus replication, or that RLV and MLV, because they are inactivated at incubator temperature (*8*), become less infective with prolonged incubation. Further experiments to define the conditions favoring or inhibiting leukemia virus replication in cells are needed.

REFERENCES

(*1*) Manaker, R. A., Strother, P. C., Miller, A. A., and Piczak, C. V.: Behavior *in vitro* of a mouse lymphoid-leukemia virus. J Nat Cancer Inst 25: 1411–1419, 1960.

(*2*) Yoshida, K., Smith K., and Pinkel, D.: Studies of murine leukemia viruses. I. Detection of Moloney and Rauscher leukemia viruses by indirect immunofluorescence. Proc Soc Exp Biol Med 121: 72–81, 1966.

(*3*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

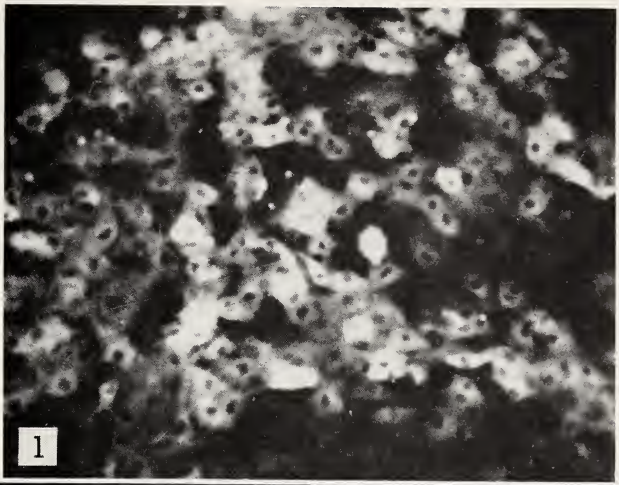
(*4*) Reed, L. J., and Muench, H.: A simple method of estimating 50 per cent endpoints. Amer J Hyg 27: 493–497, 1938.

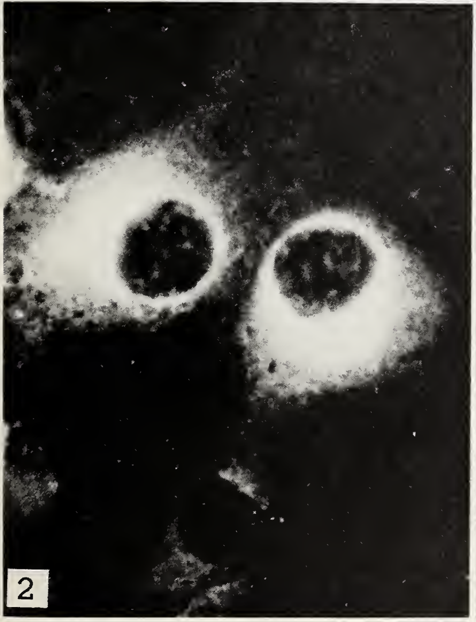
(*5*) Wheelock, E. F., and Tamm, I.: Enumeration of cell-infecting particles of Newcastle disease virus by the fluorescent antibody technique. J Exp Med 113: 301–316, 1961.

(*6*) Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J.: Complement fixation and tissue culture assays for mouse leukemia viruses. Proc Nat Acad Sci USA 53: 931–938, 1965.

(*7*) Sinkovics, J. G., and Howe, C. D.: Growth characteristics, virus yield, and interferon assay of leukemic mouse spleen tissue cultures. J Infect Dis 114: 359–372, 1964.

(*8*) Mayyasi, S.: Personal communication, 1965.





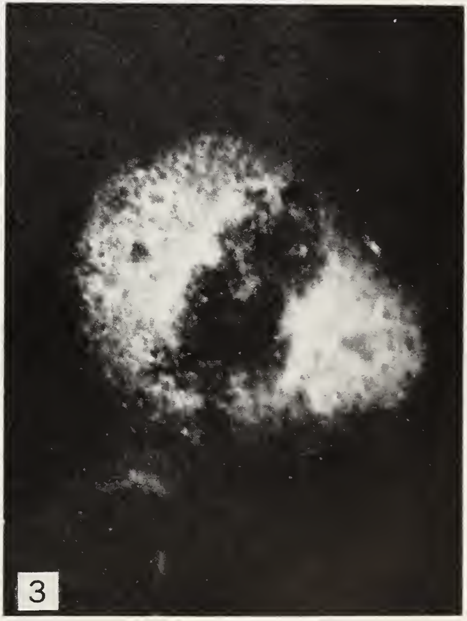


FIGURE 1.—RLV-infected cell monolayer, 10 days after inoculation of undiluted RLV plasma. Immunofluorescent cells are distributed randomly, but have a tendency to aggregate. Ultraviolet photomicrograph. × 560

FIGURE 2.—MLV-infected cell monolayer, 15 days after inoculation. Specific granular immunofluorescence is confined to cytoplasm. Ultraviolet photomicrograph. × 3,240.

FIGURE 3.—Immunofluorescent cell in mitosis, demonstrating that RLV-infected cells are capable of division and that virus antigen can be transmitted from parent cell to daughter cells. Ultraviolet photomicrograph. × 2,700

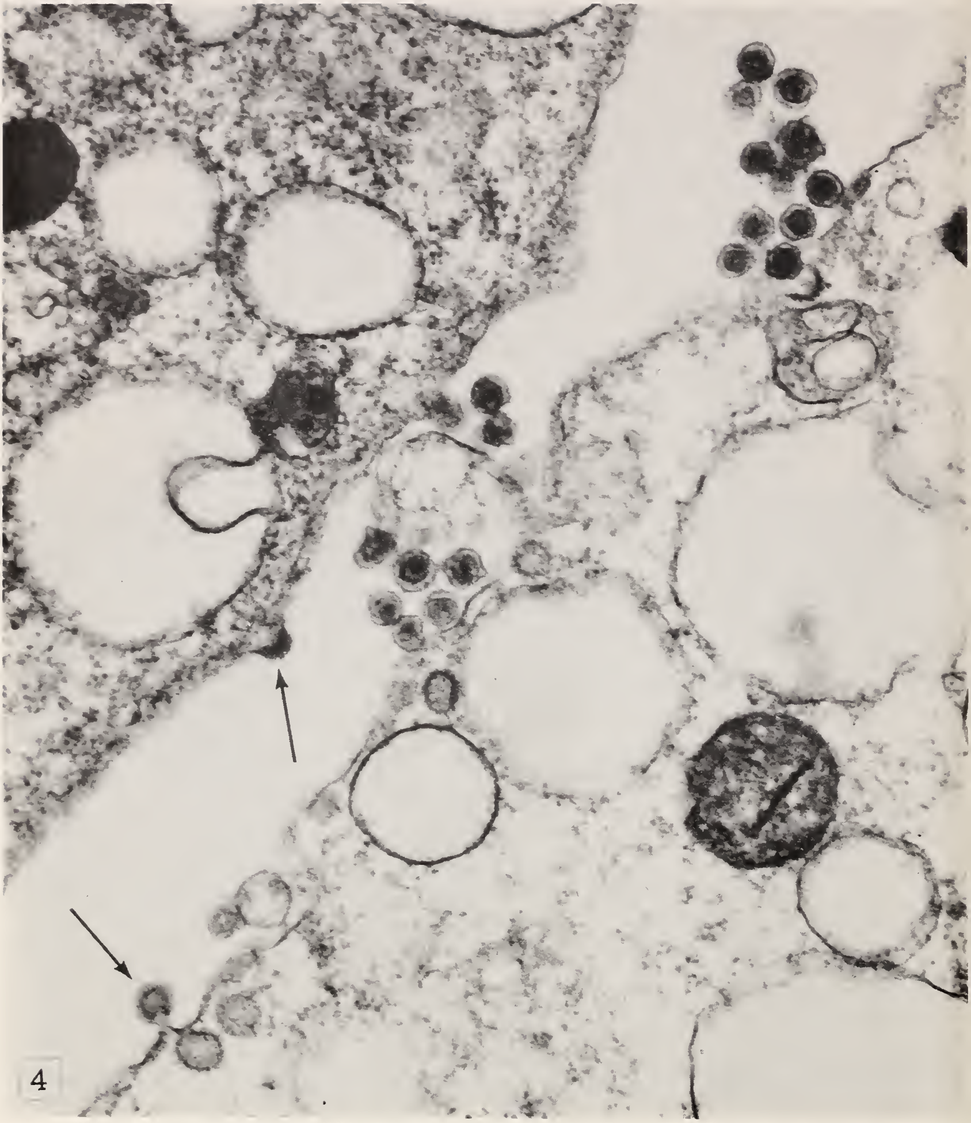


FIGURE 4.—Cells from culture depicted in figure 2 with virus particles budding from their cell membranes (*arrows*). This illustrates that immunofluorescence represents synthesis of virus as well as of virus antigen. Electron photomicrograph. Uranyl acetate and lead citrate stain. × 51,700

DISCUSSION

Dr. Sachs: Dr. Pinkel, could you tell us, in relation to the interesting results you mentioned, what was the actual titer of the virus used in your experiments?

Dr. Pinkel: We used primarily mouse plasma concentrates of virus, and we have no absolute references for the infectious titers of these preparations. Dilutions were strictly relative within these tests.

Dr. Sinkovics: Did you see nuclear fluorescence? What antibodies did the immune serum you used contain? Cytotoxic and neutralizing or only one type? Could you remove the cytotoxic antibodies with the absorptions you listed and did the neutralizing antibodies still remain in the serum?

Before Dr. Pinkel answers, I wish to comment on the tissue culture line about which I have spoken previously. When I used the cells of this line in a cytotoxic test, exposing them to antibody in the presence of guinea pig complement and eosin, not more than 10 or 12% of these cells responded with pink discoloration and swelling, indicating that only a small number of cells replicated viral antigen in this culture.

Dr. Pinkel: We did not see specific nuclear fluorescence in these studies. The difference between our results and those of others may be due to differences in methods. We used only *inbred* BALB/c mice for absorption and testing of antiviral sera and absorbed each 1 ml of antiserum with 0.1 ml of plasma once, 1 g of spleen and 0.06 g of thymus 3 times, and 1 g of liver once.

The absorbed antisera were cytotoxic for JLS-V5, JLS-V6, and JLS-V8 cells but further absorption with JLS-V6 cells removed their cytotoxic effects without removing their activity in the immunofluorescence tests. The antisera absorbed only with plasma had neutralizing properties in mouse bioassays, but we did not determine whether the further absorptions resulted in loss of neutralizing effects.

Dr. John Moloney: Dr. Pinkel, was this the material you received from Pfizer?

Dr. Pinkel: Yes.

Dr. John Moloney: Then in answer to Dr. Sachs' question, I think that material was sent to you as 10 gram equivalent material; and so he apparently made serial tenfold dilutions of the 10 gram equivalent.

Dr. Pinkel: Yes. In later experiments we exhausted the supply of Pfizer material and we used fresh plasma from mice with leukemia.

Dr. Fink: I would just like to make one comment about Dr. Sinkovics' question in relation to nuclear fluorescence. Dr. Malmgren and I in our original studies using the Rauscher virus observed fluorescence in both the nucleus and in the cytoplasm. A number of people who have heard about this paper but have not read it perhaps think we said there was only nuclear fluorescence.

We reported the nuclear fluorescence in the leukemic cells of the thymus and the lymph nodes of the infected Osborne-Mendel rat and in the virus-infected mouse ascites cell line carried in Dr. Rauscher's laboratory.

I would suggest to people who are unable to accept the observation of nuclear fluorescence that they test the same tissues we did, and also that they come to the laboratory and look at our photographs and offer another explanation if they can. The tests we did were controlled as follows: 1) The fluorescence was blocked by the previous application of anti-viral serum, but not of anti-mouse plasma serum or normal serum. Nor did the anti-mouse serum, absorbed and labeled similarly to the anti-viral serum, react with the leukemic cells. 2) The ability of the tagged antibody to stain was removed by absorption with whole spleen cells from infected mice, but not by an equal amount of splenic tissue from normal mice of the same strain. 3) An anti-egg albumin serum, prepared and labeled by a similar protocol, gave a completely negative reaction. 4) Normal tissue smears gave negative reactions with the anti-Rauscher virus-labeled antibody.

Attenuation of the Rauscher Murine Leukemia Virus Through Serial Passages in Tissue Culture [1, 2]

BRUCE S. WRIGHT *and* JENNIE C. LASFARGUES,[3] *The John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, New Jersey*

APPROXIMATELY 3 years ago, a cell line derived from a mixed culture of the spleen and thymus of normal BALB/c mice was infected with the Rauscher murine leukemia virus. In a recent report (*1*), we described in detail the continuous multiplication of the Rauscher virus by this chronically infected cell line, which we designated JLS-V5. Both newborn and weanling BALB/c mice were injected with unprocessed or concentrated cell-free tissue culture fluids. These mice developed the typical leukemia symptoms described by Rauscher (*2*). It was also reported (*1*) that after 2 years of *in vitro* passage, the JLS-V5 cell line continued to produce high concentrations of virus particles which, however, exhibited considerable reduction in infectivity for BALB/c mice. For example, a 100× cell-free tissue culture virus concentrate from the 32d passage had an ID50 of 3.5, while a similar concentrate from the 106th passage had an ID50 of 1.2 (*3*). In addition, a culture frozen at the 19th passage was found to retain its infectivity when bioassayed approximately 1 year later in BALB/c mice.

The control uninfected spleen and thymus cell line, designated JLS-V6, in early passages was negative for virus particles when concentrates of the supernatant fluids were examined under the electron microscope. However, upon serial passage, the JLS-V6 cell line had an increasing amount of virus particles, as shown by electron microscopy. This virus is morphologically similar to the Rauscher virus produced by the JLS-V5 cell line. However, at no time have any preparations or concentrates from the JLS-V6 cell line shown any leukemogenic activity in the BALB/c mice.

The progressive decrease of leukemogenic activity with a concomitant high particle count of the Rauscher virus produced by the JLS-V5 cell

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[2] Supported by Public Health Service research contract PH-43-62-144 from the National Cancer Institute.

[3] We wish to thank Dr. Frank J. Rauscher, Department of Viral Oncology, National Cancer Institute, for valuable advice.

line after prolonged passage in culture indicates a slow attenuation of the virus. This attenuation prompted investigations on the use of the live tissue culture virus as an immunizing agent against the lethal effects of Rauscher mouse plasma virus. The studies presented here describe a part of the immunological picture of the Rauscher murine leukemia as related to the JLS-V5 tissue culture virus and the possible relationship of the JLS-V6 virus to this phenomenon.

MATERIALS AND METHODS

Mice.—Weanling BALB/c mice, obtained from a colony maintained at the Pfizer Research Laboratories in Maywood, New Jersey, were used in all experiments. The origin of this strain was previously described (*1*). Unless otherwise indicated, 15 to 20 female mice (14–16 g) were caged in standard plastic boxes and supplied with water and a high-fat Wayne breeder blox feed *ad libitum.*

Viruses:

Immunizing virus

a) *Tissue culture virus JLS-V5.*—Either undiluted or concentrated cell-free tissue culture fluid from the Rauscher-virus-infected cell line was used as the immunizing agent. The cultures were grown in a modified 109 medium (*1*). The tissue culture fluid was clarified by centrifugation for 5 to 10 minutes at 1300 rpm to remove cells and cellular debris. A *single dose* of 0.1 ml of the undiluted supernatant administered intraperitoneally was used to inoculate the mice.

Concentrates of the supernatant were prepared by a procedure of differential centrifugation (*1*). Final sedimentation of the virus in the clarified supernatant was done by centrifugation at 54,000 × *g* for 90 minutes. In most cases, the pelletized virus was resuspended in a volume of 0.05 M sodium citrate required to yield a 50× or 100× concentration of the original tissue culture fluid. The concentrated virus was dispensed into glass vials and stored at −120° C.

b) *Tissue culture control JLS-V6.*—Either undiluted or concentrated cell-free tissue culture fluid from the control cell line (JLS-V6) was used for inoculating the control mice. At other times, 0.05 M sodium citrate was used. When mice were inoculated with the tissue culture fluid or its concentrate, both were prepared the same way as the JLS-V5 virus. The control mice were injected with a *single dose* of 0.1 ml intraperitoneally, with either V6 undiluted, concentrated, or 0.05 M sodium citrate solution.

Challenge virus

All of the test and control mice were challenged, at various time intervals after immunization, with 0.1 ml per mouse of a 10^{-2} dilution of a potent Rauscher mouse plasma virus given intraperitoneally. This virus dilution was prepared in 0.05 M sodium citrate. The stock challenge virus used in all the experiments (3029-191) had an infectivity titer in the order of 10^6 LD50 per ml.

EXPERIMENTS AND RESULTS

Initial Challenge Experiment With Cell-Free Tissue Culture Supernatants From JLS-V5, JLS-V6, and Their Concentrates

In July of 1964, undiluted or 50× concentrates of cell-free tissue culture supernatants from the JLS-V5 or V6 cultures at approximately the 130th passage were inoculated intraperitoneally into 8 groups of weanling BALB/c mice. In this particular experiment, the concentrates were prepared from the same type of supernatants that were inoculated as unprocessed (1×) tissue culture fluids. After 4 months, none of the mice in any of these groups had palpable spleens. They were then challenged with 0.1 ml of a 10^{-2} dilution of the standard challenge virus given intraperitoneally, which was described in the previous section. Another group of untreated weanling mice received the challenge virus only and were used as the virus controls. The results shown in table 1 were tabulated after an observation period of 216 days following the challenge virus.

None of the mice in the 2 groups originally inoculated with the 1× supernatants from the V5 cell line had palpable spleens during this observation period. However, the 2 groups inoculated with similar fluids from the V6 cell line had 75 and 88 percent splenomegaly. These two

TABLE 1.—Protection of BALB/c mice with tissue culture virus (Rauscher) against a mouse plasma challenge virus*

		Positive/total†					
		Palpable		Dead		Y50‡	
Virus	Concentration	Number	Percent	Number	Percent	Palpable	Death
Rauscher virus (V5)	1×	0/9	0	0/9	0	—	—
Control (V6)	1×	6/8	75	6/8	75	30	50
Rauscher virus (V5)	1×	0/10	0	0/10	0	—	—
Control (V6)	1×	7/8	88	5/8	63	29	42
Rauscher virus (V5)	50×	2/10	20	2/10	20	—	—
Control (V6)	50×	10/10	100	10/10	100	27	48
Rauscher virus (V5)	50×	0/9	0	0/9	0	—	—
Control (V6)	50×	6/7	86	6/7	86	30	52
Challenge virus	10^{-2}	22/22	100	21/22	95	19	45

*All mice were challenged 4 months after initial tissue culture virus.

†Observation period = 216 days after challenge with 10^{-2} plasma virus.

‡Time in days to a 50 percent response.

groups also showed 75 and 63 percent leukemic deaths, respectively, during this time.

Results obtained with the 50× concentrates of the same tissue culture fluids are also shown in this table. Mice inoculated with the V5 concentrates showed 20 and 0 percent palpations and the same leukemic deaths, respectively, in the two groups.

In contrast to these results, mice inoculated with the V6 concentrates exhibited palpations of 100 and 86 percent, respectively, with corresponding leukemic deaths.

The virus control mice that had not received any inoculation prior to the challenge virus showed 100 percent palpations and 95 percent deaths within the 216-day observation period.

It is obvious from the results of this initial experiment that the control (V6) supernatants, either concentrated 50-fold or inoculated unprocessed (1×), did not protect the mice from the challenge plasma virus. However, the Rauscher-virus tissue culture supernatants (V5) or their concentrates (50×) provided 80 to 100 percent protection of the mice when challenged with the same plasma virus.

Immunization of BALB/c Mice With 1×, 50×, and 100× Concentrates of the JLS-V5 Virus

Since encouraging results were obtained with the initial immunization experiment, a number of additional protection experiments were done with cell-free tissue culture supernatants from the JLS-V5 cell line. Three concentrations (1×, 50×, and 100×) of the immunizing virus were used. In addition, the experiments were designed with various time intervals, ranging from 1 week to 4 months between the immunizing and the challenge virus. This was done in an effort to determine the optimum protection with respect to the dose of the immunizing virus and the time interval before challenge.

Clarified cell-free tissue culture supernatants from the JLS-V5 and V6 cell lines were used as the immunizing virus (1×) for the tests shown in table 2. On two occasions 0.05 M sodium citrate, rather than the JLS-V6 cell lines were used as the immunizing virus (1×) for the tests shown that the JLS-V6 or the citrate did not offer any protection, while 71 to 92 percent protection, with respect to leukemic deaths, was observed in the test groups immunized with the JLS-V5 supernatant. As was expected, the group with the shortest time interval (1 month) between the prechallenge and challenge virus inoculations showed the least amount of protection.

Based on the mean spleen weight of the surviving mice, groups immunized with a single dose of the 1× JLS-V5 cell-free tissue culture supernatant require at least 2 and preferably *3 months* for maximum protection against the standard dilution of challenge virus.

TABLE 2.—Protection of BALB/c mice with straight tissue culture virus (1×-Rauscher virus) against a plasma challenge virus administered at various time intervals*

Virus	Interval before challenge† (months)	Positive/total							
		Palpable		Dead		Y50		Mean spleen weight	
		Number	Percent	Number	Percent	Palpable	Death	Survivors	Dead mice
Rauscher (V5)	1	16/24	67	7/24	29	28.6	—	0.854	2.876
JLS-V6 control	1	26/26	100	23/26	88	33.0	71.0	3.032	3.894
Rauscher (V5)	2	4/24	17	2/24	8	—	—	0.394	4.720
Citrate control 0.05 M	2	23/23	100	23/23	100	33	49	—	4.012
Rauscher (V5)	3	4/24	17	2/24	8	—	—	0.280	0.502
JLS-V6 control	3	27/28	96	9/28	32	44	—	2.019	1.483
Rauscher (V5)	4	8/25	32	—	—	—	—	0.302	—
Citrate control 0.05 M	4	23/23	100	2/23	9	27	—	1.648	4+

*Either citrate or 1×-JLS-V6 tissue culture fluids were given to the control groups. All mice were challenged with mouse plasma virus at 10^{-2} dilution.

†Observation period for 1 month test = 106 days after challenge virus.
" " " 2 month " = 85 " " " ".
" " " 3 month " = 52 " " " ".
" " " 4 month " = 28 " " " ".

TABLE 3.—Protection of BALB/c mice with a 50× tissue culture virus concentrate (Rauscher) against a plasma challenge virus administered at various time intervals*

		Positive/total							
	Interval before challenge† (months)	Palpable		Dead		Y50		Mean spleen weight	
Virus		Number	Percent	Number	Percent	Palpable	Death	Survivors	Dead mice
Rauscher (V5)	1	3/25	12	0/25	0	—	—	0. 472	—
Citrate control 0.05 M	1	24/24	100	23/24	96	30	46	2. 335	3. 806
Rauscher (V5)	2	1/25	4	1/25	4	—	—	0. 246	1. 835
Citrate control 0.05 M	2	24/24	100	5/24	21	31	—	1. 968	4+
Rauscher (V5)	3	11/24	46	0/24	0	22	—	0. 262	—
Citrate control 0.05 M	3	23/24	96	0/24	0	22	—	0. 531	—
Rauscher (V5)	4, not challenged	0/25	0	0/25	0	—	—	0. 135	—
Citrate control 0.05 M	4, not challenged	0/25	0	0/25	0	—	—	0. 130	—

---

*All mice were challenged with mouse plasma virus at 10^{-2} dilution.

†Observation period for 1 month test = 71 days after challenge virus.

" " " 2 month " = 42 " " " " .

" " " 3 month " = 22 " " " " .

" " " 4 month " = 108 days from tissue culture virus inoculation.

Immunization With a 50× Concentrate of the Tissue Culture Virus

The same time intervals between the prechallenge and challenge virus were used in the set of experiments shown in table 3 as were used in the previous experiments. However, a 50× concentrate of cell-free tissue culture virus from the JLS-V5 cell line was used for immunization. The control mice were inoculated with 0.05 M sodium citrate. Under these conditions, and with the same concentration of challenge virus as was used in the previous experiments, 1 and 2 months were found to be the best time interval before challenge, for maximum protection. In fact, there were no deaths in the 1-month group.

The mice in the last group of table 3 were not challenged and half of them had received the live tissue-culture virus concentrate. After approximately 4 months, both the test mice and the controls remained free of the typical Rauscher virus disease, which indicated that this concentration of the live virus has very little, if any, infectious virus.

Immunization With a 100× Concentrate of the Tissue Culture Virus

All the mice in this set of experiments were immunized with a single dose of a 100× concentrate of the cell-free tissue culture medium from the JLS-V5 cell line. The time intervals before inoculating the challenge virus ranged from 1 week to 3 months. The results at observation periods ranging from 58 to 133 days after the challenge virus are shown in table 4. A significant degree of protection (84–92%) was observed in the groups challenged as early as 1 and 2 weeks after immunization. In contrast, the controls for these two groups had a high incidence (90–100%) of leukemia.

Simultaneous Inoculation of Tissue Culture and Plasma Virus

Since the previous experiments had demonstrated that a significant protection could be noted in mice challenged only 7 days after immunization with a 100× tissue culture virus concentrate, an experiment was done to test the effect of simultaneous inoculation of the two viruses. Mouse plasma virus (Rauscher) was diluted 1:5 and 1:50 in 0.05 M sodium citrate. These diluted virus preparations were then mixed with an equal volume of either citrate (for controls) or 100× tissue culture virus. The mixtures were kept for 1½ hours at 4° C before inoculation into BALB/c mice. The final concentrations of the plasma virus, inoculated in the presence of 50× tissue culture virus, were 10^{-1} and 10^{-2}, respectively. The results, tabulated 122 days after inoculation (table 5), clearly indicate that simultaneous inoculation of tissue culture virus (50×) with either 10^{-1} or 10^{-2} dilutions of the plasma virus will not protect the mice.

TABLE 4.—Protection of BALB/c mice with a 100× tissue culture virus concentrate (Rauscher) against a plasma challenge virus administered at various time intervals*

Virus	Interval before challenge†	Positive/total							
		Palpable		Dead		Y50		Mean spleen weight	
		Number	Percent	Number	Percent	Palpable	Death	Survivors	Dead mice
Rauscher (V5)	1 week	5/25	20	4/25	16	—	—	0. 179	3. 532
Citrate control 0.05 M	1 week	18/19	95	17/19	90	25	46	0. 500	4. 578
Rauscher (V5)	2 weeks	2/24	8	2/24	8	—	—	0. 169	2. 942
Citrate control 0.05 M	2 weeks	22/22	100	22/22	100	23	50	—	3. 768
Rauscher (V5)	1 month	3/19	16	1/19	5	—	—	0. 318	4+
Citrate control 0.05 M	1 month	20/20	100	20/20	100	28	49	—	3. 636
Rauscher (V5)	2 months	10/22	45	5/22	23	—	—	0. 604	2. 545
Citrate control 0.05 M	2 months	24/24	100	19/24	79	34	48	2. 745	3. 675
Rauscher (V5)	3 months	0/24	0	0/24	0	—	—	0. 170	—
Citrate control 0.05 M	3 months	22/22	100	4/22	18	33	—	2. 593	4+

*All mice were challenged with mouse plasma virus at 10^{-2} dilution.

†Observation period for 1 week test = 133 days after challenge virus.
" " " 2 week " = 126 " " " ".
" " " 1 month " = 130 " " " ".
" " " 2 month " = 72 " " " ".
" " " 3 month " = 58 " " " ".

TABLE 5.—Inability of the tissue culture virus to protect BALB/c mice when inoculated simultaneously with the plasma virus

Virus	Final dilution of plasma virus	Positive/total						
		Palpable		Dead		Y50		Mean spleen weight of survivors at sacrifice
		Number	Percent	Number	Percent	Palpable	Death	
Plasma virus + JLS-V5 virus	10^{-1}	13/13	100	13/13	100	22	35	—
Plasma virus + 0.05 M Na citrate	10^{-1}	15/15	100	15/15	100	22	39	—
Plasma virus + JLS-V5 virus	10^{-2}	12/14	86	12/14	86	32	48	0. 180
Plasma virus + 0.05 M Na citrate	10^{-2}	12/13	92	10/13	77	33	54	1. 950

TABLE 6.—Time required to protect mice with a live 50× tissue culture virus concentrate (Rauscher) against a mouse plasma challenge virus*

Virus	Interval before challenge† (days)	Positive/total						Mean spleen weight of survivors at sacrifice
		Palpable		Dead		Y^{50}		
		Number	Percent	Number	Percent	Palpable	Death	
Rauscher (V5)	7	0/20	0	0/20	0	—	—	0. 198
Citrate control 0.05 M	7	18/20	90	1/20	5	34	—	1. 343
Rauscher (V5)	5	5/20	25	—	—	—	—	0. 496
Citrate control 0.05 M	5	16/18	89	3/18	17	40	—	2. 639
Rauscher (V5)	3	17/18	94	12/18	67	44	50	2. 538
Citrate control 0.05 M	3	16/16	100	11/16	69	41	49	3. 199
Rauscher (V5)	1	17/22	77	1/22	5	41	—	1. 570
Citrate control 0.05 M	1	14/20	70	5/20	25	38	—	1. 132
Rauscher (V5)	0	11/14	79	10/14	71	29	37	0. 431
Citrate control 0.05 M	0	14/16	88	12/16	75	28	42	0. 675
Rauscher (V5)	+1	9/20	45	4/20	20	—	—	0. 523
Citrate control 0.05 M	+1	5/16	31	—	—	—	—	0. 640

*All mice were challenged with mouse plasma virus at 10^{-2} dilution.

†Observation periods
7 days = 44 day
5 " = 57 "
3 " = 66 "
1 " = 44 "
0 " = 66 "
+1 " = 57 "

Determination of the Shortest Time Necessary to Protect BALB/c Mice With a Tissue Culture Virus Concentrate

Since it was shown that a 100× concentrate of the JLS-V5 tissue culture virus would protect the mice if they were challenged 7 days after they were immunized and that simultaneous inoculation of the two viruses would not offer any protection, an additional experiment was done to determine the minimum time required to demonstrate protection with a single dose of a 50× concentrate of the tissue culture virus. Groups of mice were immunized 0, 1, 3, 5, and 7 days before they were challenged with the standard dilution of the plasma virus. In addition, another group was inoculated with the challenge virus first and 24 hours later with the tissue culture virus. The results shown in table 6 demonstrate the lack of protection by the tissue culture virus if it was inoculated either after the challenge virus (day +1) or simultaneously (day 0). In addition, similar results were obtained if the immunized mice were challenged within 3 days. However, protection was observed if the time interval before challenge was extended to 5 and 7 days.

Inability of Control Tissue Culture Concentrates to Protect BALB/c Mice

As shown in previous experiments, a single inoculation of 1× or 50× concentrates of JLS-V6 tissue culture supernatants will not protect BALB/c mice against a challenge of the mouse plasma virus. An additional experiment was then carried out in which mice were immunized with a series of inoculations of either a concentrate of the control cell virus (JLS-V6) or the Rauscher virus from JLS-V5. In this experiment, mice were immunized by a series of 3 weekly injections of 100× concentrates of tissue culture supernatants from either of the two cell lines and challenged 2 weeks after the last inoculation with the mouse plasma virus. Sodium citrate (0.05 M) was used for the control groups, which also received 3 weekly inoculations. As can be seen from the results shown in table 7, there was no protection of the mice inoculated with the JLS-V6 concentrate, while a similar immunization schedule with the live JLS-V5 Rauscher virus concentrate resulted in a 91 percent protection.

The lower portion of table 7 shows the results of a single inoculation of a JLS-V6 concentrate (100×) into newborn BALB/c mice. Here again, the viral agent did not produce a demonstrable disease within an observation period of 169 days.

Demonstration of Live Virus in a JLS-V5 Concentrate

Since no deliberate attempt was made to inactivate the JLS-V5 virus used in the immunization experiments, efforts were made to demonstrate residual live virus in a JLS-V5 concentrate. In this test, a 100× concen-

TABLE 7.—Protection of BALB/c mice with a live virus concentrate (100×) of JLS-V5 and inability of a control JLS-V6 concentrate (100×) to protect mice against a challenge of mouse plasma virus (Rauscher)*

		Positive/total							
		Palpable		Dead		Y50		Mean spleen weight	
Virus	Interval before challenge†	Number	Percent	Number	Percent	Palpable	Death	Survivors	Dead mice
(100×) JLS–V5	Series of 3 weekly injections followed 2 weeks later with the challenge virus	2/22	9	2/22	9	—	—	0. 163	3. 025
Citrate control 0.05 M	Series of 3 weekly injections followed 2 weeks later with the challenge virus	14/18	78	12/18	67	40	52	0. 676	3. 643
(100×) JLS–V6	Series of 3 weekly injections followed 2 weeks later with the challenge virus	14/14	100	14/14	100	26	49	—	3. 517
Citrate control 0.05 M	Series of 3 weekly injections followed 2 weeks later with the challenge virus	14/14	100	14/14	100	27	58	—	3. 719
	JLS–V6 100× concentrate in newborn BALB/c mice								
(100×) JLS–V6	Mice were not challenged	0/13	0	0/13	0	—	—	0. 121	—
Citrate control 0. 05 M	Mice were not challenged	0/13	0	0/13	0	—	—	0. 097	

*All mice were challenged with mouse plasma virus at 10^{-2} dilution.

†Observation period for 100×—JLS-V5 weanling mice = 71 days after challenge virus
" " " 100×—JLS-V6 " " = 141 " " " " .

Observation period for newborn mice that received 100×—JLS-V6 = 169 days.

trate (3025-155-2) of JLS-V5 was further concentrated tenfold to 1,000×. A similar preparation of the JLS-V6 agent was made from a pool of two 100× concentrates (3034-180-1 and 3034-262-1) and each of the 1,000× preparations was inoculated into 20 weanling BALB/c mice. All the mice inoculated with the JLS-V5 concentrate became palpable with a mean time to palpation of 31 days (table 8). After 4 months, 71 percent of these mice had died with the typical Rauscher virus infection. The mean time to leukemic death was 70 days. The average spleen weight of the five surviving mice was 2.5 g. In contrast to these results, none of the mice inoculated with the 1,000× JLS-V6 concentrate were palpable during an observation period of 122 days. These latter results confirm those reported earlier on the JLS-V6 agent in newborn mice. However, by the use of a 1,000× concentrate of the JLS-5 virus, it was possible to demonstrate some residual Rauscher virus activity.

DISCUSSION

According to the definitions of attenuation, the studies reported here indicate that the Rauscher murine leukemia virus in the JLS-V5 cell line has become attenuated through serial passages in tissue culture. However, it is evident that the attenuated virus still retains some leukemogenic activity for the BALB/c mice. This was noticed only with the highly concentrated preparations of the virus and therefore suggests the presence of a very low proportion of infectious virus mixed together with a high concentration of noninfectious virus. This explanation would be consistent with the uniformly high electron microscope counts. However, since it is not known just how many leukemia virus particles constitute one LD50 upon bioassay in mice, it is possible that only a small proportion of the JLS-V5 cells is producing the leukemogenic virus and that this concentration is sufficient to immunize the mice but not produce the disease. This possibility would be consistent with the observation that a shorter time interval is required between the prechallenge and challenge virus, as the concentration of the former increases. The fact that simultaneous inoculation of the tissue culture and plasma virus will not protect the mice, and that 5 days were required before protection was demonstrable, might indicate that the tissue culture virus has to multiply within the host mice to a threshold value to effectively interfere with the challenge virus. During this time interval, neutralizing antibody might be formed and keeps the infectious virus concentration below that which is necessary to produce leukemic death. It is interesting to note that the minimum time necessary to demonstrate protection is approximately the same as that which Rauscher observed for the plasma virus to be first detectable in infected mice (*4*).

Formalinized vaccines have been reported for leukemias of the Friend and Rauscher type. Friend (*5*) demonstrated significant protection of Swiss mice when the latter were immunized with formalin-treated filtrates

Table 8.—Demonstration of residual Rauscher virus activity in a 1,000× concentrate of JLS-V5 and lack of leukemogenic response to a similar control concentrate (JLS-V6)

Virus	Concentration	Electron microscope count*	Positive/total						
			Palpable		Dead		Y50		Mean spleen weight of survivors at sacrifice
			Number	Percent	Number	Percent	Palpable	Death	
JLS-V5	1, 000×	TNTC/sq	17/17	100	12/17	71	31	70	2. 493
JLS-V6	1, 000×	100–500/sq	0/20	0	0/20	0	—	—	—

*TNTC/sq = virus particles were too numerous to count on a 200-mesh electron microscope grid.

Observation period for JLS-V5 mice = 133 days.

Observation period for JLS-V6 mice = 122 days.

of leukemic spleens and challenged with the active virus. Fink and Rauscher (*6*) obtained similar results with BALB/c mice and formalin-treated cell-free Rauscher virus from leukemic spleens. Mayyasi and Bulfone (*7*) also reported that formalin-inactivated Rauscher virus from the JLS-V5 cell line effectively protected BALB/c mice against the active mouse plasma virus. Recently, Barski and Youn (*8*) reported the effectiveness of using multiple injections of the JLS-V5 cells (frozen and thawed 3 times) to protect BALB/c mice against a lethal dose of a splenic preparation of the Rauscher virus.

Regardless of the explanation for the immunity observed, be it an interferon, viral interference, true immunization of the mice with subsequent development of neutralizing antibodies, or any combination, our studies indicate the possibility of using single or multiple injections of live cell-free tissue culture virus from long-term cultures of the JLS-V5 cell line to induce immunity in BALB/c mice and prevent the development of the Rauscher murine leukemia.

Work is in progress to define the duration of the immunity resulting from vaccination and to determine the exact nature of this immunity.

SUMMARY

During the last 3 years, the Rauscher murine leukemia virus has been maintained in an established cell line derived from the cells of the spleen and thymus of normal weanling BALB/c mice. Cell-free virus, from early passages of the infected cell line, produced the typical Rauscher virus disease in weanling BALB/c mice. However, decreased leukemogenic activity without loss of high yields of virus particles was observed from later passages of this infected cell line. The early passage culture held in the deepfreeze at the nineteenth passage has retained its infectivity. BALB/c mice inoculated with preparations of the late-passaged virus, either as unprocessed cell-free tissue culture fluids or their concentrates, proved highly resistant to a subsequent challenge with lethal concentrations of the mouse plasma virus. Protection depended on the concentration of the immunizing virus and the time interval before challenge with the plasma virus. This time interval was at least 5 days when a single injection of a 50× concentrate of the virus was used as the immunizing dose. Simultaneous inoculation of tissue culture and plasma virus did not protect the mice. Multiple injections of this attenuated Rauscher virus from tissue culture protected 91 percent of the immunized mice. Similar immunization with the control cell virus did not protect the mice. This control virus does not produce any demonstrable disease in newborn BALB/c mice and will not protect weanling mice as the Rauscher virus from the infected culture does. Thus, live virus from the JLS-V5 cells can be used to immunize BALB/c mice against a lethal challenge of the plasma virus. Within limits, concentrates of this virus can also be used

as the immunizing agent; however, highly concentrated virus preparations produce the typical Rauscher virus disease in BALB/c mice.

REFERENCES

(*1*) Wright, B. S., and Lasfargues, J. C.: Long-term propagation of the Rauscher murine leukemia virus in tissue culture. J Nat Cancer Inst 35: 319–327, 1965.

(*2*) Rauscher, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J Nat Cancer Inst 29: 515–543, 1962.

(*3*) Reed, L. J., and Muench, H.: A simple method of estimating 50 per cent endpoints. Amer J Hyg 27: 493–497, 1938.

(*4*) Rauscher, F. J., and Allen, B. V.: Growth curve of a murine leukemia virus in mice. J Nat Cancer Inst 32: 269–275, 1964.

(*5*) Friend, C.: Immunological relationships of a filterable agent causing a leukemia in adult mice. J Exp Med 109: 217–228, 1959.

(*6*) Fink, M. A., and Rauscher, F. J.: Immune reactions to a murine leukemia virus. I. Induction of immunity to infection with virus in the natural host. J Nat Cancer Inst 32: 1075–1082, 1964.

(*7*) Mayyasi, S. A., and Bulfone, L. M.: The immune response to the Moloney and Rauscher viruses propagated in tissue culture. Proc Amer Soc Microbiol, p 109, 1965.

(*8*) Barski, G., and Youn, J. K.: Immunization against Rauscher mouse leukemia with tissue culture material. Science 149: 751–752, 1965.

DISCUSSION

Dr. Stansly: This seems to be a very interesting system to determine the relationship between the various virus strains.

Dr. Wright: We have some experiments in progress now to determine whether there are any cross reactions.

Dr. Friend: Have you inoculated the V6 into newborn mice?

Dr. Wright: Yes, we have.

Dr. Friend: Is it infective?

Dr. Wright: No.

Dr. Friend: Were your control animals of the same age as your vaccinated ones?

Dr. Wright: Yes. Newborn mice were inoculated with either sodium citrate or a 100×concentrate of the V6. After 169 days, mice inoculated with the V6 concentrate, but not challenged, had an average spleen weight of 0.121 g, while the average for those receiving the citrate was 0.097 g.

Dr. Barski: Have you tried to use the cells or cell fractions in your experiment?

Dr. Wright: No, we have not, not yet.

Dr. Barski: What is the electron microscope morphology of the virus particles in the V6? Have you tried to infect the V6 line with Rauscher virus?

Dr. Wright: The V6 particles look exactly the same as the Rauscher virus. In fact, I understand that even in the density gradient they band at 1.16. I have not tried to reinfect it, but Dr. Tyndall tells me he did and he was successful.

Dr. Bianco: I wonder whether the pretreatment with V6 would also induce resistance against transplantation of leukemic cells. Do you have any information on this?

Dr. Wright: I haven't tried to challenge with the cells. Most of my experiments have been with cell-free virus.

Dr. Sinkovics: Did you detect any antibodies in the blood of the protected mice?

Dr. Wright: Yes, we did.

Leukemia Virus Release in Chemically or Physically Induced Lymphomas in BALB/c Mice [1, 2]

Rodolfo Ribacchi, *M.D., and* Gaetano Giraldo, *M.D., Division of Cancer Research, University of Perugia, Perugia, Italy*

In recent years many leukemia viruses have been isolated from organs of mice with spontaneous leukemia, belonging to strains with a high (*1*, *2*) or low (*3*, *4*) leukemia incidence, and from some lines of epithelial and connective-type transplanted tumors (*5–8*).

Some of these viruses, isolated from different strains of mice and under different biological conditions, are not identical but differ in some biological and immunological characteristics (*9*).

More recently, active leukemia viruses also have been isolated from organs of mice with a low incidence of spontaneous leukemia, in which leukemia had been induced by methylcholanthrene (*4*), dimethylbenzanthracene (*10*), urethan (*11*), and ionizing radiation (*12*, *13*).

Two years ago, we began a long-term study in an attempt to confirm the results relative to the isolation of leukemia viruses from organs of mice with physically or chemically induced leukemia and to ascertain whether leukemia viruses, activated spontaneously or isolated from chemically or physically induced leukemia in the same strain of inbred mice (BALB/c), are identical or differ in some characteristics.

MATERIALS AND METHODS

Leukemogenic agents.—The chemical carcinogens chosen were urethan in aqueous solution and 9,10-dimethyl-1,2-benzanthracene (DMBA) [3] in a 1% colloidal gelatin suspension (*14*); X rays were the physical agents.

Four groups of mice were inoculated subcutaneously with urethan in doses of 1, 2, 5, and 10 mg. The first 2 groups received a single dose and

---

[1] Presented at the Conference on Murine Leukemia, Philadelphia, Penna., October 13–15, 1965.

[3] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.
from the National Cancer Institute.

[3] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

the remaining groups, fractionated doses of 1 mg each at intervals of 4 to 5 days.

DMBA was injected subcutaneously into 2 groups of mice in doses of 50 and 100 μg, respectively.

Two other groups of mice were irradiated with 400 r and 500 r, respectively, in fractionated doses of 100 r each at intervals of 4 to 5 days.

Mice.—BALB/c inbred mice of both sexes were used, and all received the first or single treatment with the chemical or physical agent within the first 24 hours of life.

At weaning, the mice of each group were allowed to breed normally for the duration of the experiment. All the females treated with urethan produced two or more litters, but most of those treated with DMBA or with ionizing radiation remained sterile (*15–17*).

Control mice: In the BALB/c mice of both sexes bred in the animal colony of the University of Perugia, Division of Cancer Research, from 1957 to date, the incidence of lymphoid tumors of spontaneous origin was 3.2% at an average age of 558 days, and exactly 3.3% in males and 3% in females normally bred at the average ages of 633 and 484 days (*18*).

Virus preparations.—Virus preparations were made by Moloney's method from tissue homogenates (thymus, spleen, lymph nodes, liver, and brain) of mice carrying a malignant lymphoma (*19, 20*). For each chemically or physically induced lymphoma, three or more viral extractions were prepared separately. The concentration of the viral preparations was expressed in grams-equivalent.

As a source of spontaneously activated virus, a fifth transfer generation of a BALB/c lymphosarcoma was cultured in Eagle's basal medium to which 0.5% lactalbumin hydrolysate and 10% calf serum were added (*21*).

Newborn BALB/c mice, less than 24 hours old and rarely older, were injected subcutaneously or intraperitoneally with viral preparations. Those still alive at weaning were allowed to breed normally for the duration of the experiment.

RESULTS

Leukemogenesis by Physical or Chemical Agents in BALB/c Mice

Table 1 shows the incidence and the average age of lymphoid tumors induced by urethan, DMBA, and X rays.

In mice treated at birth with urethan, the incidence of lymphoid tumors increased from 6.2 to 18.3% as the dose of carcinogen administered was increased. The average age at tumor appearance tended, on the contrary, to diminish from 394 to 231 days.

No difference in the incidence or average age of lymphomas was detected between the 2 groups of mice treated with 5 and 10 mg of urethan.

TABLE 1.—Incidence and average age of malignant lymphomas induced by chemical and physical agents in BALB/c mice treated when newborn

Leukemogenic agents	Dose	Survivors at weaning	With malignant lymphoma			Without malignant lymphoma	
			Number	%	Average age	Number	Average age
Urethan	1 mg	32	2	6.2	394	30	397
	2 mg	29	3	10.3	284	26	405
	5 mg*	32	6	18.3	231	26	355
	10 mg*	31	6	19.3	238	25	343
DMBA	50 μg	50	9	18.0	224	41	293
	100 μg	50	16	32.0	163	34	292
X rays	400 r*	61	31	50.8	202	30	499
	500 r*	19	12	63.1	179	7	478

*Fractionated doses at intervals of 4 to 5 days.

Mice treated with 50 and 100 μg of DMBA developed lymphoid tumors with a frequency of 18 and 32%, respectively, at the average ages of 224 and 163 days.

In the 2 groups of mice irradiated with 400 r and 500 r, the incidence of lymphoid tumor appearance was 50.8 and 63.1%, respectively, at the average ages of 202 and 179 days.

Both in urethan-treated mice and in those irradiated, the incidence of lymphoid tumors showed no sex variation (*15, 16*).

Morphologically, lymphoid tumors induced by chemical or physical agents may be divided into two groups: lymphomas localized exclusively or mainly in the thymus (lymphosarcomas) and lymphatic leukemias.

Cell-Free Transmission of Transplantable, Spontaneous Lymphosarcomas and of Chemically or Physically Induced Lymphomas

Transplanted lymphosarcoma (*table 2*).—Newborn BALB/c mice were inoculated with tissue culture fluid from a transplanted lymphosarcoma, at the fifth transfer generation, which arose spontaneously in a BALB/c mouse. Of the 4 mice still alive at weaning, 3 died with lymphoid tumors, giving an incidence of 75% at an average age of 281 days. A viral concentrate was prepared from 1 of these 3 mice; Moloney's method was used for the second passage. In the three subsequent passages, lymphoid tumors arose in all the mice injected, at a progressively decreasing average age.

Urethan-induced lymphoma (*table 3*).—To show the presence of an active leukemia virus in the organs of BALB/c mice with urethan-induced lymphomas, 5 viral extractions were prepared separately from 5 different mice with a urethan-induced lymphoma. In 4 of the 5 groups of mice injected intraperitoneally or subcutaneously with the extracts, reticular

TABLE 2.—Cell-free transmission of a transplantable lymphosarcoma (fifth transfer generation) spontaneously developed in a BALB/c mouse

Passage	Viral concentration (g:ml)	Dose (ml)	Route of inoculation	Survivors at weaning	With reticular tumors			Without reticular tumors	
					Number	%	Average age	Number	Average age
1	TCF*	0. 1	IP†	4	3	75. 0	281	1	524
2	2. 5:1	0. 1	IP	11	11	100. 0	166	—	—
3	2:1	0. 1	IP	3	3	100. 0	118	—	—
4	2:1	0. 1	IP	4	4	100. 0	144	—	—

*TCF: tissue culture fluid.
†IP: intraperitoneal.

tumors arose with a variable incidence of from 28.5 to 100%, at an average age of 361 to 502 days.

Of the 31 mice inoculated with different extracts, 17 (54.8%) died with reticular tumors at an average age of 437 days, 8 died without developing reticular tumors at an average age of 376 days, and 6 are still alive at an average age of 526 days.

Data relative to the incidence and the age, at appearance of the reticular tumors, of the mice belonging to the two following serial passages are not yet complete, since many of the mice are still alive.

DMBA-induced lymphoma.—Three viral preparations at the concentrations shown in table 4 were made separately from some BALB/c mice with DMBA-induced lymphoma.

In 2 of the 3 groups of mice inoculated intraperitoneally with the viral extracts, reticular tumors arose with an incidence of 33.3 and 80%, respectively, at the average ages of 586 and 431 days. The second group of mice, in which reticular tumors did not appear, had been inoculated at the age of 3 days.

Of the 20 mice inoculated with the three viral extracts, 6 (30%) died with reticular tumors at the average age of 483 days, 8 died without reticular tumors at the average age of 535 days, and 6 are still alive at the average age of 654 days.

X-ray-induced lymphoma.—Four viral extracts, at concentrations shown in table 5, were prepared separately from some mice with lymphoma induced by ionizing radiation. In 3 of the 4 groups inoculated subcutaneously with the extracts, reticular tumors arose with a frequency varying from 25 to 55.5% at average ages between 365 and 515 days. Some of the mice are still alive.

Of the 31 BALB/c mice inoculated with the viral extracts, 10 (32.2%) died with reticular tumors at the average age of 428 days, 15 died without reticular tumors at the average age of 425 days, and 6 are still alive at the average age of 665 days.

TABLE 3.—Cell-free transmission of urethan-induced malignant lymphomas in BALB/c mice

Passage	Bioassays	Viral concentration (g:ml)	Dose (ml)	Route of inoculation*	Survivors at weaning	With reticular tumors			Without reticular tumors		Alive	
						Number	%	Average age	Number	Average age	Number	Average age
1	A	1:1	0. 1	IP	10	8	80. 0	484	2	242	—	—
	B	3:1	0. 1	SC	5	5	100. 0	367	—	—	—	—
	C	2:1	0. 1	SC	4	2	50. 0	502	2	543	—	—
	D	6:1	0. 1	IP	5	—	—	—	2	428	3	586
	E	1:1	0. 1	IP	7	2	28. 5	361	2	292	3	467
Total					31	17	54. 8	437	8	376	6	526
2	B	2:1	0. 1	IP	10	7	70. 0	263	1	221	2	363
3	B	2:1	0. 1	IP	5	1	20. 0	212	—	—	4	212

*IP: intraperitoneal; SC: subcutaneous.

TABLE 4.—Cell-free transmission of DMBA-induced malignant lymphomas in BALB/c mice

Passage	Bioassays	Viral concentration (g:ml)	Dose (ml)	Route of inoculation*	Survivors at weaning	With reticular tumors			Without reticular tumors		Alive	
						Number	%	Average age	Number	Average age	Number	Average age
1	A	4:1	0.05	IP	6	2	33.3	586	2	568	2	661
	B	2:1	0.1	IP	9†	—	—	—	5	530	4	651
	C	3:1	0.1	IP	5	4	80.0	431	1	488	—	—
Total					20	6	30.0	483	8	535	6	654

*IP: intraperitoneal.

†Mice were inoculated with viral extract when 3 days old.

TABLE 5.—Cell-free transmission of X-ray-induced malignant lymphomas in BALB/c mice

Passage	Bioassays	Viral concentration (g:ml)	Dose (ml)	Route of inoculation*	Survivors at weaning	With reticular tumors			Without reticular tumors		Alive	
						Number	%	Average age	Number	Average age	Number	Average age
1	A	3:1	0.1	SC	7	3	42.8	491	4	566	—	—
	B	2:1	0.1	SC	8	2	25.0	515	5	371	1	672
	C	2:1	0.1	SC	7	—	—	—	2	363	5	664
	D	3.5:1	0.1	SC	9	5	55.5	365	4	391	—	—
Total					31	10	32.2	428	15	425	6	665
2	A	1:1	0.1	IP	8	3	37.5	231	1	334	4	342

*SC: subcutaneous; IP: intraperitoneal.

TABLE 6.—Morphology of reticular tumors induced in BALB/c mice injected at birth with cell-free preparations

Viral agents	Passage No.	Mice with reticular tumors	Lymphoid tumors			Myeloid leukemia			Reticulum cell sarcoma		
			Number	%	Average age	Number	%	Average age	Number	%	Average age
S-LV*	1	3	3	100. 0	281	—	—	—	—	—	—
	2	11	11	100. 0	166	—	—	—	—	—	—
	3	3	3	100. 0	118	—	—	—	—	—	—
	4	4	4	100. 0	144	—	—	—	—	—	—
U-LV†	1	17	11	64. 7	424	2	11. 7	439	4	23. 5	474
	2	7	5	71. 4	253	1	14. 2	290	1	14. 2	290
DMBA-LV‡	1	5	2	40. 0	393	2	40. 0	606	1	20. 0	398
X-RAY-LV§	1	8	5	62. 5	352	1	12. 5	335	2	25. 0	547
	2	3	3	100. 0	231	—	—	—	—	—	—

*S-LV = Spontaneous activated leukemia virus.
†U-LV = Urethan activated leukemia virus.
‡DMBA-LV = DMBA activated leukemia virus.
§ X-RAY-LV = radiation activated leukemia virus.

Data relative to the incidence of reticular tumors at the second passage are not yet complete, since some of the animals are still alive.

Morphology of reticular tumors developed in BALB/c mice injected at birth with cell-free preparations (*table 6*).—All reticular tumors developing in BALB/c mice injected with the spontaneously activated leukemia virus (S-LV) were of the lymphoid type.

Mice receiving viral extracts prepared from primary urethan-, DMBA-, and radiation-induced lymphomas developed lymphoid tumors and (at a lower incidence) myeloid leukemias and reticulum cell sarcomas. In addition, the incidence of lymphoid tumors at the second passage is increasing and has reached 100% in mice treated with radiation activated leukemia virus (X-RAY-LV) but their average age is decreasing.

DISCUSSION

From the above data, it is possible to state that in BALB/c mice with urethan-, DMBA-, and X-ray-induced lymphomas, as well as in mice carrying a transplanted lymphosarcoma, active leukemia viruses are present. Similar results have been obtained in other low leukemia strains of mice, namely, C3H (*22*), Swiss (*10*), ICI/SPF (*11*), C57BL (*12*), RF (*4, 13*), and ICR (*23*) treated with different leukemogenic agents (ionizing radiation, methylcholanthrene, dimethylbenzanthracene, urethan, and 4-nitroquinoline *N*-oxide).

It is not, however, as easy to understand the mechanism of viral activation. Chemical and physical leukemogenic agents might, indeed, activate a latent leukemogenic virus, and this would become the agent responsible for the production of lymphomas. On the other hand, the leukemic state produced by the chemical and physical agents might be responsible for the viral activation.

As far as the problem of similarities and differences between viruses spontaneously activated and viruses isolated from mice of the same strain with lymphoid tumors induced by different chemical and physical agents is concerned, it is not yet possible to provide any satisfactory answer.

In fact, in mice inoculated with viral extracts prepared from urethan-, DMBA-, and X-ray-induced lymphomas, reticular tumors arose from three different cellular lines: lymphoid, myeloid, and reticular. At the first passage, lymphoid tumors developed with a greater incidence and at a lower average age than for other types of tumors. At the second passage as well, the incidence of lymphoid tumors increased in relation to that of myeloid leukemias and reticulum cell sarcomas. It is therefore to be expected that in the subsequent passages only lymphoid tumors would develop. Thus, the biological criterion is insufficient to demonstrate possible differences between the above isolated viruses. We are working on an immunological method that will provide a comprehensive answer to this question.

SUMMARY

To study leukemia-virus release in chemically or physically induced lymphomas in mice, and to ascertain whether leukemic viruses, activated spontaneously or isolated from chemically or physically induced lymphomas in the same strain of inbred mice, are identical or differ in some characteristics, BALB/c mice were treated when newborn with urethan, DBMA, and X rays.

Following Moloney's method, viral extracts were prepared from chemically or physically induced lymphomas and were injected into newborn mice less than 24 hours old.

The incidence of reticular tumors induced by cell-free preparations was 54.8, 30, and 32.2% in mice injected with viral extracts obtained from urethan-, DMBA-, and X-ray-induced lymphomas at the average ages of 437, 483, 428 days, respectively.

The spontaneously activated leukemia virus was isolated from a fifth transfer generation of a transplanted lymphosarcoma that developed in a BALB/c mouse.

Mice injected with spontaneously activated leukemia virus developed only lymphoid tumors, whereas mice inoculated with viral extracts prepared from urethan-, DMBA-, and X-ray-induced lymphomas developed lymphoid tumors and, at lower incidence, also myeloid leukemias and reticulum cell sarcomas.

No satisfactory answer is yet available about differences and/or similarities among those BALB/c leukemia viruses.

REFERENCES

(*1*) GROSS, L.: "Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK-leukemic extracts or AK-embryos. Proc Soc Exp Biol Med 76: 27–32, 1951.

(*2*) ———: Viral (egg-borne) etiology of mouse leukemia; filtered extracts from leukemic C58 mice, causing leukemia (or parotid tumors) following inoculation into newborn C57 brown or C3H mice. Cancer 9: 778–791, 1956.

(*3*) PRECERUTTI, A., and LAW, L. W.: Isolation of a murine leukemogenic virus P-LLV. Nature (London) 198: 801–803, 1963.

(*4*) IRINO, S., OTA, Z., SEZAKI, T., SUZAKI, M., and HIRAKI, K.: Cell-free transmission of 20-methylcholanthrene-induced RF mouse leukemia and electron microscopic demonstration of virus particles in its leukemic tissue. Gann 54: 225–237, 1963.

(*5*) MOLONEY, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J Nat Cancer Inst 24: 933–951, 1960.

(*6*) FRIEND, C.: Leukemia of adult mice caused by a transmissible agent. Ann NY Acad Sci 68: 522–532, 1957.

(*7*) STANSLY, P. G., and SOULE, H. D.: Experiment on the transplantation and cell-free transmission of a reticulum cell sarcoma in BALB/c mice. Proc Amer Assoc Cancer Res 3: 270, 1961.

(*8*) Graffi, A.: Chloroleukemia in mice. Ann NY Acad Sci 68: 540–558, 1957.

(*9*) Moloney, J. B.: The murine leukemias. Fed Proc 21: 19–31, 1962.

(*10*) Toth, B.: Development of malignant lymphomas by cell-free filtrates prepared from a chemically induced mouse lymphoma. Proc Soc Exp Biol Med 112: 873–875, 1963.

(*11*) Salaman, M. H., and Flocks, J.: Attempt to isolate a leukemogenic virus from urethan-induced leukemia in mice. Brit Empire Cancer Campaign for Research, forty-second annual report, 1964.

(*12*) Lieberman, M., and Kaplan, H. S.: Leukemogenic activity of filtrates from radiation induced lymphoid tumors in mice. Science 130: 387–388, 1959.

(*13*) Parsons, D. F., Upton, A. C., Bender, M. A., Jenkins, V. K., Nelson, E. S., and Johnson, R. R.: Electron microscopic observation on primary and serially passed radiation-induced myeloid leukemia of the RF mouse. Cancer Res 22: 728–736, 1962.

(*14*) Pietra, G., Spencer, R., and Shubik, P.: Response of newly born mice to a chemical carcinogen. Nature (London) 183: 1689, 1959.

(*15*) Ribacchi, R., Milia, U., and Giraldo, G.: Tumori linfoidi de uretano in topi BALB/c/Cb/Se substrain. Lav Anat Pat Perugia 24: 125–134, 1964.

(*16*) Ribacchi, R., Milia, U., Paoletti, I., Giraldo, G., and Jantorni, P.: Leucemogenesi da radiazioni ionizzanti in topi meonati del BALB/c/Cb/Se substrain. Lav Anat Pat Perugia 25: 47–55, 1965.

(*17*) Ribacchi, R., and Giraldo, G.: Tumori ovarici e mammari in topi BALB/c/Cb/-Se substrain trattati alla nascita con 9:10-dimethyl-1:2-benzanthracene (DMBA). Lav Anat Pat Perugia 24: 195–202, 1964.

(*18*) Squartini, F., and Rossi, G.: Alta incidenza di leucemie nei topi BALB/c allattati da RIII. Atti di un Simposio Internazionale, Pp 129–148. I. Virus nella Leucemia dei Mammiferi. Accademia Nazionale dei Lincei, Roma, 1964.

(*19*) Ribacchi, R., Giraldo, G., and Milia, U.: Trassmissione acellulare dei tumori linfoidi da uretano in topi BALB/c/Cb/Se substrain. Lav Anat Pat Perugia 24: 135–143, 1964.

(*20*) ———: Isolamento di un virus leucemico da topi del BALB/c/Cb/Se substrain con leucemia da radiazioni ionizzanti. Law Anat Pat Perugia 25: 83–96, 1965.

(*21*) Ribacchi, R., and Giraldo, G.: Isolamento di un virus leucemico da un linfosarcoma trapiantabile del BALB/c/Cb/Se substrain. Lav Anat Pat Perugia 25: 101–109, 1965.

(*22*) Gross, L.: Attempt to recover filterable agent from X-ray-induced leukemia. Acta Haemat Jap 19: 353–361, 1958.

(*23*) Kinosita, R., and Tanaka, T.: Lymphoma in ICR mice treated with 4-nitroquinoline *N*-oxide (4-HQO). *In* viruses, Nucleic Acids, and Cancer. University of Texas M. D. Anderson Hospital and Tumor Institute, Baltimore, Williams & Wilkins Co., 1963.

Papers presented at the Conference for which no manuscripts were submitted.

A Study on the Infectivity of Rauscher Virus by Bioassay and Electron Microscopy
J. P. Levy and M. Boiron

Irradiation of Tumor Viruses
Raymond Latarjet

U.S. GOVERNMENT PRINTING OFFICE:1966

LIBRARY
NIH
Amazing Research.
Amazing Help.

http://nihlibrary.nih.gov

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080

NIH LIBRARY
3 1496 00026 3643

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service